12-2010

Stress-induced targeting of molecular chaperones in the yeast Saccharomyces cerevisiae

Hugo Tapia

STRESS-INDUCED TARGETING OF MOLECULAR CHAPERONES IN THE YEAST *SACCHAROMYCES CEREVISIAE*

by

Hugo Tapia

APPROVED:

_____

Advisor, Kevin A. Morano, Ph.D.

_____

Michael Blackburn, Ph.D.

_____

Peter J. Christie, Ph.D.

_____

Michael C. Lorenz, Ph.D.

_____

Ambro van Hoof, Ph.D.

APPROVED:

_____

George Stancel, Ph.D. Dean, The University of Texas Graduate School of Biomedical Sciences at Houston

STRESS-INDUCED TARGETING OF MOLECULAR CHAPERONES IN THE YEAST *SACCHAROMYCES CEREVISIAE*

A

DISSERTATION

Presented to the Faculty of

The University of Texas Health Science Center at Houston and

the University of Texas M.D. Anderson Cancer Center

Graduate School of Biomedical Sciences

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

by

Hugo Tapia

Houston, TX

August 2010

Acknowledgements

I would like to acknowledge my advisor, Dr. Kevin Morano, for his guidance and constant support. He took what was a very naïve person and helped me become a less naïve scientist. Kevin has helped me grow as a writer, a speaker and importantly as an independent scientific researcher. Beyond his role as an advisor, Kevin has been a good friend with his door always open to talk about science or any kind of idea that might possibly be rolling around in my head at the moment. I am heartily thankful to Kevin for all of his help during my graduate career.

I would also like to thank all of the current and former lab members that I have had the chance to work with during the years including Patrick Gibney, Lance Shaner, Amy Trott, Yanyu Wang, Jacob Verghese, Jennifer Abrams, Kimberly Cope, Davin Ng, Theresa Bui and David Vu. I would especially like to thank Pat and Lance for all the help when I first joined the lab and for making the lab a really fun place to be.

I would like to thank the faculty, staff and students of the Department of Microbiology and Molecular Genetics for all of the helpful scientific discussions and quick help with any type of administrative concern. Special thanks to Dr. Aaron Carman and Dr. Jesus Eraso who were there for many of my random ramblings and helped me grow as a scientist. My heartfelt thanks to everyone at MMG whose names I did not mention, but who contributed in any form towards the successful completion of my dissertation. I would like to specially thank the members of my supervisory committee consisting of Dr. Michael Lorenz, Dr. Ambro van Hoof, Dr. Peter Christie and Dr. Michael Blackburn for advice and guidance.

Finally, I would like to thank my family and friends. I would like to thank my sister Magaly for being a constant source of joy and laughter in my life. Thanks to all of my aunts, uncles and cousins, who have given me unwavering support. I would also like to thank the friends that I have made here in Houston that have made this time even more enjoyable. To conclude, I would like to thank my mom; thanks to her I have become the person that I am today. Muchas gracias Mamá.

STRESS-INDUCED TARGETING OF MOLECULAR CHAPERONES IN THE YEAST *SACCHAROMYCES CEREVISIAE*

Publication No. _____

Hugo Tapia

Advisor: Kevin A. Morano

The eukaryotic stress response is an essential mechanism that helps protect cells from a variety of environmental stresses. Cell death can result if cells are not able to properly adapt and protect themselves against adverse stress conditions. Failure to properly deal with stress has implications in human diseases including neurodegenerative disorders and distinct cancers, emphasizing the importance of understanding the eukaryotic stress response in detail. As part of this response, expression of a battery of heat shock proteins (HSP) is induced, which act as molecular chaperones to assist in the repair or triage of unfolded proteins.

The 90-kDa HSP (Hsp90) operates in the context of a multi-chaperone complex to promote the maturation of nuclear and cytoplasmic clients. I have discovered that Hsp90 and the co-chaperone Sba1 accumulate in the nucleus of quiescent *Saccharomyces cerevisiae* cells in a karyopherin-dependent manner. I isolated nuclear accumulation-defective *HSP82* mutant alleles to probe the nature of this targeting event and identified a mutant with a single amino acid substitution (I578F) sufficient to prevent nuclear accumulation of Hsp90 in quiescent cells. Diploid *hsp82-I578F* cells exhibited pronounced defects in spore wall construction and maturation, resulting in catastrophic sporulation. The mislocalization and sporulation phenotypes were shared by another previously identified *HSP82* mutant allele, further linking localization to Hsp90 functional status. Pharmacological inhibition of Hsp90 with macbecin in sporulating diploid cells also blocked spore formation, underscoring the importance of this chaperone in this developmental program.

The yeast molecular chaperone Hsp104 is a member of the Hsp100 superfamily of AAA+ ATPases. Unlike the Hsp90 family of chaperones, Hsp104 is not restricted to a specific set of client proteins, but rather assists in reactivating stress-denatured proteins by solubilizing protein aggregates. I have discovered that Hsp104, along with the Hsp70 chaperone, Ssa1, and the sHSP Hsp26 accumulate into RNA processing bodies (P-bodies) and stress granules, sites of mRNA metabolism. I found that Hsp104 recruits both Ssa1 and Hsp26 to P-bodies and that these three chaperones are required for stress granule formation. These findings suggest a possible role for chaperones in mRNA metabolism by aiding in the assembly, disassembly or conversion of these enigmatic mRNP complexes. Taken together, the work presented in this dissertation serves to better understand the eukaryotic stress response by illustrating the importance of subcellular-chaperone localization in key biological processes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1: Introduction to the heat shock response and molecular chaperones in the yeast *Saccharomyces cerevisiae*

Introduction and Background

Conservation of the cellular stress response among all domains of life – All cells have acquired the ability to sense and quickly adapt to stress present in their ever-changing environments. Cells are under constant attack from a variety of stresses such as extreme temperature fluctuations, starvation, chemical stressors and radiation. Despite the variety of stresses cells might encounter, life has evolved to withstand and actually prosper under stressful conditions. The cellular stress response, conserved from bacteria to humans, helps cells not only sense stress but also ultimately recover from stress conditions. Stressed cells adapt by remodeling their metabolic activities, ceasing to grow and by expressing a set of dedicated stress response genes. Stress response genes have been found in all three domains of life: bacteria, archae and eukarya (1-3). Notwithstanding the divergence between these organisms, the cellular stress response involves expression of many shared proteins conserved throughout life including: molecular chaperones, protein degradation machinery, DNA repair enzymes, redox regulators as well as cell cycle regulators (3). The conservation of this response indicates that it must have originated early during evolution, in order for cells to survive the barrage of insults present in their ever-changing environment, and for life to develop and continue evolving into more complex life forms.

The types of stresses cells encounter in their environment can quickly change. It is interesting to note that when encountering a stressor such as heat shock, the cellular stress response allows cells to acquire protection against other distinct stressors, such as starvation (4). This cross-protection likely helps cells be prepared for any types of future stress that a cell might encounter while already in a vulnerable state. The cellular stress

response is a common response regardless of the source of stress, making the study of any specific stress, for example starvation, relevant to the overall stress response occurring with a multitude of different stressors such as heat shock, osmotic stress, radiation, oxidative damage or pathogen stress, in any organism. To this end, I have used the model yeast, *Saccharomyces cerevisiae*, to study how cells deal with conditions of stress related to the lack of nutrients. Further, I specifically investigated how molecular chaperones; proteins involved in binding stabilizing and assisting in the refolding of unfolded polypeptides, change subcellular localization in response to conditions of starvation.

The heat shock response in S. cerevisiae – The yeast *S. cerevisiae* grows optimally at 30°C, at which temperature it can undergo a complete cell cycle in approximately 90 minutes. When yeast cells are stressed with temperatures above 30°C, they induce the heat shock response, or HSR (5). The HSR in yeast is a model for generalized stress responses due to the highly conserved actions that arise due to different types of stressors. At high temperatures, yeast cells transiently arrest growth in the G1 phase of the cell cycle (6). During this transient growth arrest, cells induce the expression of a multitude of genes with a wide range of functions required for protection against cytoxic stress (4). Genes expressed during stress response include molecular chaperones, metabolic proteins, (such as trehalose-producing enzymes), cell wall remodeling proteins and protein degradation factors (2, 5). Molecular chaperones protect newly translated proteins, as wells as preventing thermolabile proteins from unfolding and aggregating, which occurs at high temperatures. Once proteins become unfolded by heat, chaperones bind to exposed hydrophobic residues, normally buried in a properly folded protein, and

prevent aggregation with other denatured proteins. Molecular chaperones have the additional role of controlling the quality of denatured proteins, delivering unfolded proteins damaged beyond repair to the proteosome for degradation, a common occurrence due to the excess of unfolded and aggregated proteins that result from high temperatures. Molecular chaperones help proteins reach a homeostatic balance, a concept recently thought of as protein homeostasis, or proteostasis (7).

Transcriptional activation of the heat shock response in yeast is controlled by three transcription factors, Hsf1, Msn2 and the partially redundant Msn4 (5). Hsf1, highly conserved among all eukaryotes, is encoded by the *HSF1* gene in yeast. *HSF1* is an essential transcription factor needed under all conditions tested, highlighting its role during 'normal' non-stress biology as well (5). Although Hsf1 is thought to be the primary transcription factor governing the heat shock response, it is also implicated in different cellular functions such as pathogen resistance, longevity and proteostasis (5, 8, 9). Hsf1 possesses a helix-turn-helix DNA binding domain, a leucine zipper trimerization domain, and a C-terminal transcriptional activation domain (10). Hsf1 binds as a homotrimeric complex to conserved heat shock element (HSE) motifs found in the promoter regions of target genes containing three inverted repeats with the sequence 5'-nGAAn-3' (11). The number of HSE and spaces between each HSE determine the strength and kinetics of induction for specific genes during heat shock activation. It is still unclear how the cell senses and is activated by thermal stress, however, Hsf1 is highly regulated by different post-translational events including phosphorylation, de-phosphorylation, acetylation, as well as being repressed during normal growth conditions by a group of molecular chaperones (12). Msn2/4 are a set of highly homologous zinc-

finger transcription factors that respond to more general stressors, and therefore activate the general stress response (5). Msn2/4 regulate the expression of approximately 200 genes in order to help cells deal with stressors such as heat shock, osmotic shock, oxidative stress, low pH, starvation, and high ethanol concentrations (4). Msn2/4 bind to conserved STRE motifs found in the promoter regions of target genes with the sequence 5'-CCCCT-3' (4). *MSN2* is a constitutively expressed transcription factor, while expression of *MSN4* is dependent itself on Msn2/4 (4). While both Hsf1 and Msn2/4 transcription factors control overlapping aspects of the yeast stress response, very little overlap exists between the set of genes induced by both factors. Some chaperones, including *HSP12, HSP26* and *HSP104* are among the genes whose expression is controlled by both transcription factors (5).

Both Hsf1 and Msn2/4 transcription factors are capable of inducing the expression of molecular chaperones. When cells encounter stresses such as heat shock, thermolabile proteins can unfold and aggregate, with detrimental consequences to the cell. In general, molecular chaperones are responsible for promoting proper protein folding to a native conformation while also preventing unfolded proteins from misfolding and aggregating. Molecular chaperones recognize stretches of hydrophobic amino acids that become exposed when proteins unfold due to denaturing stresses and provide the unfolded proteins with an adequate environment where they can refold into their native state. Different types of molecular chaperones have evolved and have been maintained through evolution to deal with protein misfolding and aggregation by contrasting mechanisms (Table 1-1). The functional divergence between molecular chaperones is remarkable, with some chaperones merely holding unfolded proteins ("holdases"), while

others can actively bind and release unfolded polypeptides in ATP-dependent cycles to promote protein refolding. The work described in this dissertation will focus on chaperones and co-chaperones associated with the Hsp90 and Hsp100 chaperone families.

Table 1-1. Yeast molecular chaperones

Type	Yeast protein	Cellular compartment	Function
Hsp90	Hs(c/p)82	Cytosol/Nucleus	Signaling protein chaperone
Hsp100	Hsp104	Cytosol/Nucleus	Protein dissagregation/degradation (Bacterial ClpB homolog)
Hsp70	Ssa1-4	Cytosol/Nucleus	Protein folding chaperone
	Ssb1-2	Cytosol	Ribosome associated native protein folding chaperone
	Ssc1	Mitochondrial matrix	Organelle specific protein folding chaperone
	Kar2	ER lumen	Organelle specific protein folding chaperone
	Ssz1	Cytosol	Ribosome associated Ssb co-chaperone
Small Hsps	Hsp26/Hsp42	Cytosol/Nucleus	Unfolded protein holding chaperones
Hsp110	Sse1-2	Cytosol/Nucleus	Hsp70 nucleotide exchange factor
Hsp60	Hsp60	Mitochondrial matrix	Organelle specific chaperonin
Hsp40	Ydj1	Cytosol/Nucleus	Hsp70 activating protein
	Sis1	Cytosol/Nucleus	Hsp70 activating protein
Chaperonins	Cct1-8	Cytosol	Protein folding chaperone

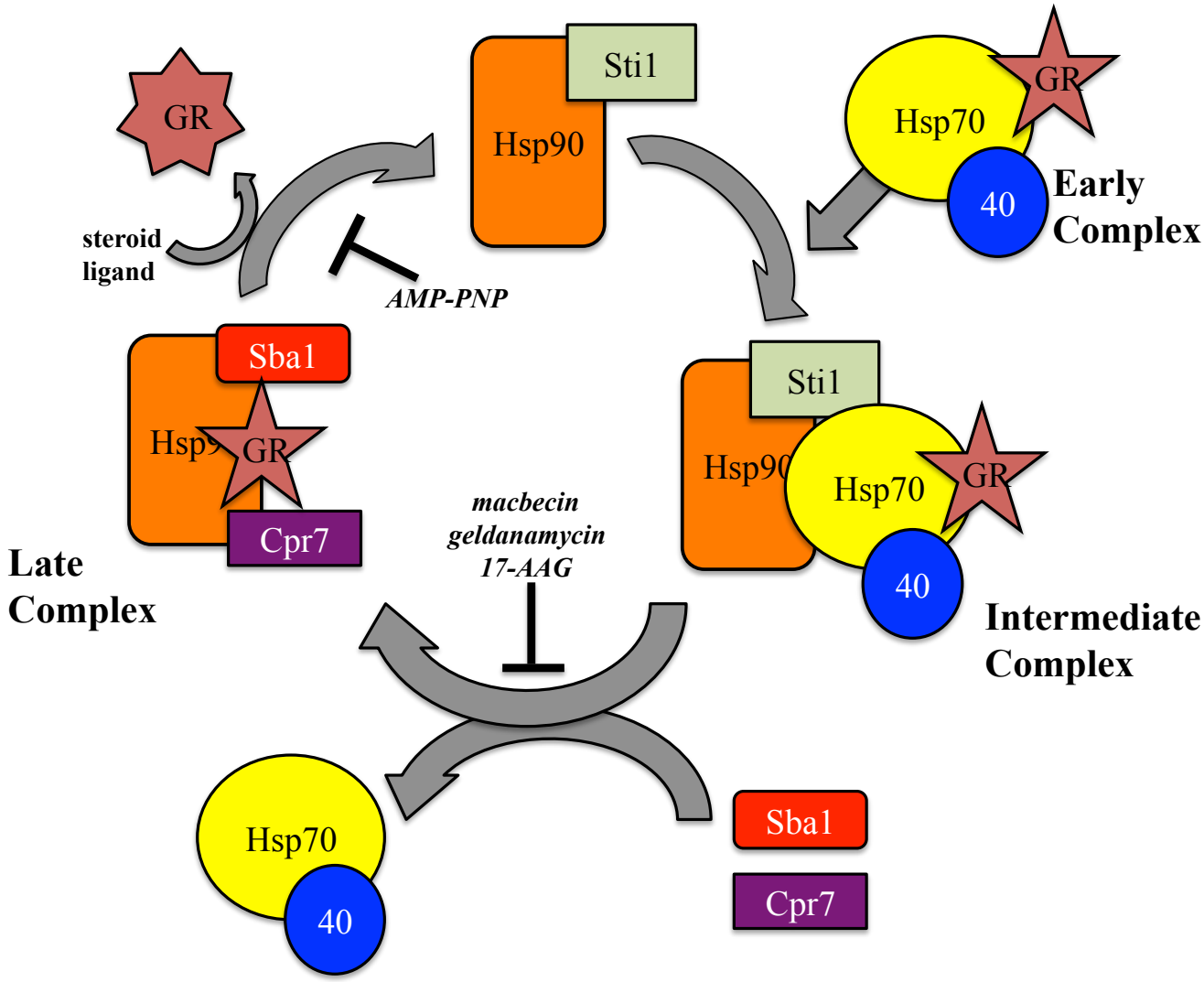
The Hsp90 family of molecular chaperones – The Hsp90 family of molecular chaperones is highly conserved throughout all three domains of life. Unlike chaperones that assist with bulk protein folding, Hsp90 participates in the folding and maturation of a distinct set of 'client' proteins (13). The list of proteins that are clients of Hsp90 is extensive, and they are generally involved in cellular signaling. Multiple important signal transduction proteins require Hsp90 and associated co-chaperones for their functional maturation, stability and regulation (13-16). Many Hsp90 client proteins are of importance to human health due to their implications in disorders such as Alzheimer's disease (beta-amyloid fibers, Tau), and some cancers (v-Src, p53) (17-22). Recently, Hsp90 has emerged as an important mediator of evolution, being able to modulate traits in yeast, flies, and plants as well as antibiotic resistance in fungal pathogens (23-26). In *Saccharomyces cerevisiae*, two homologs of Hsp90 exist, *HSP82* and *HSC82*, sharing approximately 90% sequence identity and together encoding 1-2% of the total protein in the cytosol (27). *HSP82* is expressed at low levels in unstressed cells but is highly induced during stress. *HSC82* is constitutively expressed at an abundant level, yet is only induced 2-3 fold during stress (28). Deletion of either Hsp90 gene leads to no detectable phenotype, but double deletion of both is lethal at all temperatures (28). Members of the Hsp90 family function as dimers, and like many other molecular chaperones, protein folding activity is driven by its intrinsic ATPase activity (29). Binding of ATP to the dimerized Hsp90 N-terminus induces a conformation change in both monomers leading to transient dimerization of the N-terminal nucleotide-binding domain resulting in regulation of client proteins (29).

Proper function of Hsp90 requires formation of a multichaperone complex including a variety of other chaperones and co-chaperones that regulate substrate transfer

Figure 1-1. The yeast Hsp90 chaperone cycle

The model client protein, glucocorticoid receptor (GR) is depicted as a star. Before delivery to Hsp90, GR is bound to Hsp70 and its co-chaperones in the "early complex". GR is delivered to Hsp90 through the actions of Hsp70 and its co-chaperones. The "intermediate complex" consists of ATP-bound Hsp90 and co-chaperones. Clients are transferred from Hsp70 to Hsp90 followed by the release of "intermediate stage" co-chaperones and binding of "late stage" co-chaperones. Finally, the "late complex" consists of ATP-bound Hsp90 and co-chaperones, maintain GR in a ligand-binding competent state. Ligand binding to GR is coupled with Hsp90 ATP hydrolysis and matured client protein release as well as co-chaperone release. Shown in italics are compounds able to inhibit Hsp90 cycle progression at different stages.

Figure 1-1. The yeast Hsp90 chaperone cycle



from the Hsp70 chaperone system (Figure 1-1). A set of co-chaperones has been identified in yeast and other organisms that regulate Hsp90 both positively and negatively. One common method by which these partner proteins interact with Hsp90 is through a tetratricopeptide repeat (TPR) domain present in co-chaperones that interact with the C-terminal EEVD sequence present in Hsp90 as well as Hsp70 (30). The most well studied TPR-containing protein is Sti1 (homolog of mammalian Hop), which bridges the interaction between Hsp90 and Hsp70 (31). While bridging Hsp90 and Hsp70, Sti1 also inhibits Hsp90s ATPase activity, allowing for client transfer from Hsp70 to Hsp90 (31). Furthermore, Sba1 (yeast homolog to mammalian p23) is a co-chaperone found in the late stage chaperone cycle of Hsp90 (32). Sba1 binds to the amino terminal nucleotide binding domains of both Hsp90 molecules in the functional dimer and stabilizes the chaperone in the ATP-bound state (33). Another group of Hsp90 co-chaperones are the immunophilins (Cpr6/7 in yeast), which are peptidyl-prolyl cis/trans isomerases (PPIases), found to bind the C-terminal end of Hsp90, replacing Sti1 in late stage complexes (34). Another late stage co-chaperone is Cdc37, which plays a critical role in activating cyclin-dependent protein kinases, and works both with Hsp90 and independently to help kinases mature properly (35). Finally, a small group of co-chaperones, including Aha1 and Hch1, function as late stage co-chaperones stimulating Hsp90's ATPase activity (15). Along with this large of dedicated Hsp90 co-chaperones, Hsp70 and its dedicated co-chaperones, are important not only in presenting client proteins to Hsp90, but are also required to regulate Hsp90 function with a diverse set of clients (15).

Hsp90 is one of the most abundant proteins in the cytosol of eukaryotic cells, and its abundance is increased further by stress (28). While Hsp90 orthologs such as TRAP-1 and Grp94 are restricted to the mitochondrial matrix and endoplasmic reticulum, respectively, little is known about the subcellular localization of cytoplasmic Hsp90 (36, 37). Hsp90 is known to shuttle between the cytoplasm and the nucleus when in complex with immature steroid receptor substrates (e.g. glucocorticoid receptor, androgen receptor) (38). Hsp90, alongside its late stage co-chaperones (TPR-domain immunophilins, FKBP52) and the client protein, shifts its localization from being predominantly in the cytoplasm to the nucleus upon activation by signaling molecules such as glucocorticoid and testosterone (38). Once in the nucleus Hsp90 and its client protein disassociate (38). In *S. cerevisiae*, Hsp90 exhibits uniform localization throughout the cytoplasm and nucleus during vegetative growth, consistent with involvement in both cytoplasmic and nuclear processes (39). Before the studies presented in this dissertation, it was unknown whether this distribution is altered under conditions of cellular stress.

The Hsp100 family of molecular chaperones – The yeast molecular chaperone Hsp104, along with its bacterial homolog caseinolytic peptidase B (ClpB), are members of the Hsp100 superfamily of AAA+ ATPases (ATPase associated with diverse cellular activities) (40). Unlike the Hsp90 family of chaperones, Hsp104 does not fold any specific set of 'client' proteins, but rather assists in reactivating stress-denatured proteins by solubilizing protein aggregates. Hsp104 has the ability to solubilize almost any protein that becomes aggregated after denaturing stress, an activity conserved in fungi, bacteria, plants, and eukaryotic mitochondria, but oddly absent from metazoan

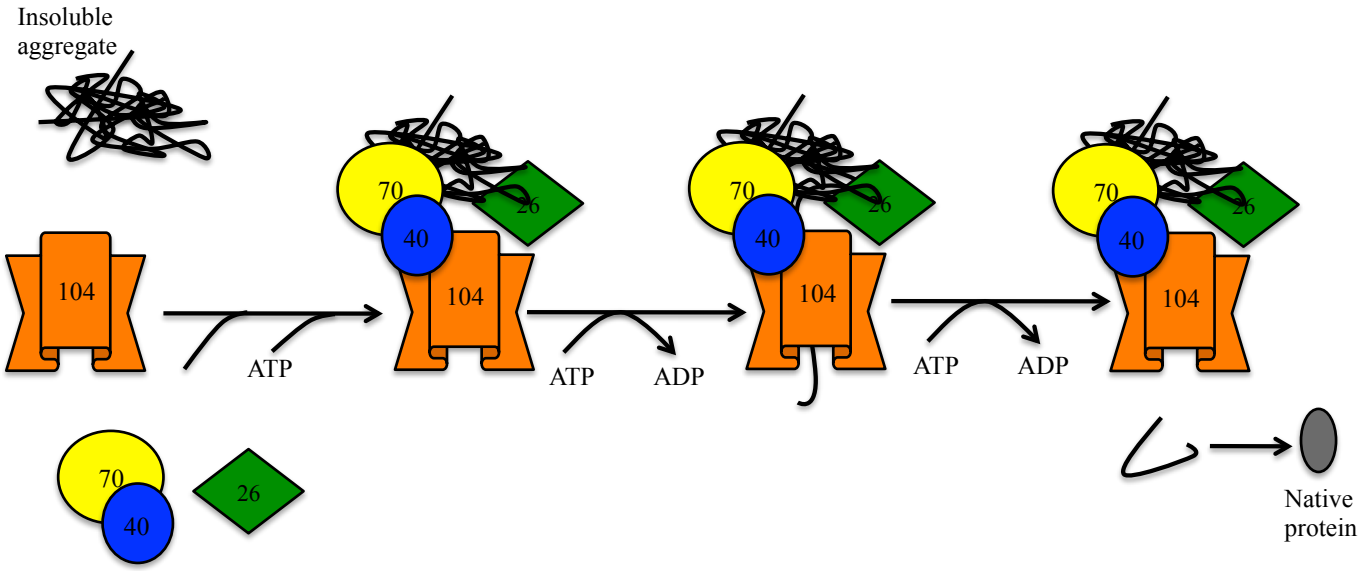
cytoplasms (41-43). Expression of Hsp104 is highly induced upon stress but is at low levels under normal growth conditions (44). Transcriptional regulation of Hsp104 is independently mediated by the stress responsive transcription factors Hsf1 and Msn2/4, which bind to two HSE and three STRE elements in the *HSP104* promoter (45). Hsp104 has attracted much attention due to its role in thermotolerance, defined as the cells ability to withstand a sudden lethal temperature shock (5). Cells can acquire thermotolerance to lethal heat stress when presented with a milder heat stress by inducing expression of heat shock proteins that will aid in restoring activity to heat inactivated proteins (46). Over-expression of Hsp104 alone is sufficient to allow cells to be thermotolerant without the preconditioning treatment (46).

Although not required for normal growth, Hsp104 can increase survival 100- to 1000- fold when cells are exposed to extreme heat or other harsh stresses (44, 47). For many protein disaggregating activities, Hsp104 acts in cooperation with the Hsp70 family of chaperones and co-chaperones to extract polypeptide chains from protein aggregates and facilitate their refolding (Figure 1-2) (48, 49). The function of yeast Hsp104 is genetically dependent on *HSP70* (50). A yeast strain with deletion in both genes shows a greater decrease in thermotolerance than either single gene deletion alone (50). Further, deletion of *HSP104* leads to the overexpression of Hsp70, yet this only partially restores thermotolerance (50). In addition, even though Hsp104 is not essential under normal growth conditions, it turns out to be essential when Hsp70 levels are reduced, suggesting that Hsp104 and Hsp70 are functionally related (50). Similar observations in vitro demonstrate that Hsp104 functions closely with the Hsp70/40 chaperones (51). When each purified chaperone was added in equal concentrations to urea-denatured firefly

Figure 1-2. Dissagregation by Hsp104 in combination with Hsp70 and Hsp26

The Hsp70 chaperone system and the sHSP Hsp26 interact with Hsp104 and act before or in conjuction with Hsp104. Hsp70/Hsp26 likely assists in presenting substrates to Hsp104 and Hsp70 might also coordinate ATP hydrolysis by Hsp104. Hsp104 performs the primary work of dissagregation. Polypeptides are removed from the aggregate by forced translocation through the Hsp104 central channel and released as an unfolded protein to refold with assistance of other chaperones, which might include Hsp70.

Figure 1-2. Dissagregation by Hsp104 in combination with Hsp70 and Hsp26



luciferase (FFL), refolding was observed in 30-60 minutes in the presence of ATP. This refolding activity required the cooperation of each chaperone, since none of the chaperones tested exhibited the extent of refolding activity individually (48).

Small heat shock proteins (sHSPs), such as Hsp26, have also been shown to aid in the clearance of aggregates by Hsp104 (52). Hsp26 assembles with misfolded proteins and bolsters access of the disaggregation machinery, composed of Hsp104/Hsp70, to the aggregated proteins (52). Hsp26 is highly induced as cells enter quiescence or when they are subjected to heat stress (53). *In vitro,* Hsp26 forms oligomeric structures and assembles into large super-complexes with aggregation-prone proteins when stressed with heat (54). Hsp26, along with other sHSPs, such as Hsp42, aids in the refolding of these aggregation-prone proteins by holding them in a semi-competent state, where Hsp104 can carry out reactivation of the denatured proteins, with assistance from the Hsp70 chaperone system (52). Beyond the interactions with Hsp70 and sHSPs, Hsp104 interacts with proteins containing TPR domains such as Sti1 and Cpr7 (55). Even though the relevance of these interactions is not yet clear, it has been suggested that Sti1 and Cpr7 might be involved in sorting substrates after they are released by Hsp104 (55).

When inactive, Hsp104 is thought to exist in either monomeric, dimeric, or trimeric forms (56). Once activated, Hsp104 assembles into a hexameric ring structure (57). Aggregated proteins are unfolded and subsequently threaded through this hexameric ring in order to become reactivated (46). The state of Hsp104 ATP/ADP binding regulates substrate binding, with ADP bound states having low and ATP bound state having high affinities for unfolded proteins (58). Formation of the hexameric ring is

also dependent on intrinsic ATP hydrolysis regulated by two nucleotide-binding domains (NBDs) within Hsp104 (57).

The Hsp104 molecular chaperone plays a central role in the propagation of yeast amyloids called prions (59). Prions are conformationally modified proteins that form proteinaceous infectious particles (60). Prions are known to cause a number of neurodegenerative diseases in mammals such as Scrapie in sheep, 'mad cow disease' in cows and Creutzfeldt-Jakob disease in humans (60). Although not present in mammals, the effect of Hsp104 has been studied in yeast models of human prion diseases such as Huntington disease and Creutzfeldt-Jakob disease (61). Overexpression of yeast *HSP104* leads to an extended lifespan of transgenic Huntington mice and a reduction of polyglutamine aggregation and toxicity in mammalian cells (61).

Despite the large body of knowledge surrounding chaperone function, many questions remain to be answered. A detailed investigation into the localization patterns of Hsp90 and Hsp104 and their co-chaperones has not been described. Along with denaturing proteins, different stresses have also been described to alter the intracellular localization of proteins (62, 63). The work described in this dissertation seeks to assess the localization patterns of major molecular chaperones during conditions of stress.

Significance of the study

Cells must be prepared to deal with an assortment of different insults that are present in their ever-changing environment. Molecular chaperones play a vital role in cells by promoting proteostasis; preventing protein aggregation and maintaining proteins in an active state (7). Molecular chaperones play an important role in both the proper folding of proteins as well as delivering unfolded proteins damaged beyond repair to the proteosome for degradation. A variety of diseases such as Creutzfeldt-Jakob, Alzheimer's disease, and Hungtington's disease are caused by the aggregation of proteins in amyloid plaques, resulting in cell death or other destabilizing pathologies (7). Increasing our knowledge of chaperone biology can help advance the development of treatments for such diseases by either enhancing or diminishing the function of endogenous molecular chaperones. Knowledge obtained from studying chaperone function using an efficient and inexpensive model system, like yeast, can be applied to mammalian cells in the future. In this study, using primarily fluorescent microscopy techniques, I have been able to link chaperone function to important cellular functions such as development and mRNA metabolism in yeast. Insights gained in this study can later be applied to mammalian systems.

When I began this study, very little was known about the subcellular distribution of Hsp90 during normal conditions or during conditions of stress. It was previously described that the Hsp90 molecular chaperone is able to shuttle between the cytoplasm and the nucleus in mammals when in complex with immature steroid receptor substrates. Chapter 3 describes a new localization pattern for the yeast Hsp90 molecular chaperone based on nutrient availability. I show that Hsp90, and its late stage co-chaperone Sba1,

accumulate in the nucleus of quiescent cells using a karyopherin-dependent mechanism. I also show that this nuclear accumulation is not a general stress response but is specific to glucose depletion with Hsp90 translocating to the nucleus in response to progression through the diauxic shift into quiescence. Using error-prone PCR I constructed non-accumulating *HSP82* alleles in order to better dissect the role of Hsp90 in the nucleus of quiescent cells. A single amino acid substitution of isoleucine 578 to phenylalanine in the carboxy terminus of the protein was sufficient to block nuclear accumulation of Hsp90 upon glucose exhaustion. Importantly, I show that lack of Hsp90 nuclear accumulation leads to a pronounced lag phase in the transition from quiescence to logarithmic growth as well as an overall slower growth rate, suggesting a possible defect in the re-entry into the cell cycle from quiescence.

Chapter 4 expands on the investigation of Hsp90 nuclear accumulation during quiescence and demonstrates that Hsp90 activity and localization are required for proper spore development in yeast. I show that Hsp90 localizes to the nucleus of all four meiotic spores. Using a mutant allele (I578F) that fails to localize to the nucleus upon glucose exhaustion, we show that this mutant allele also fails to accumulate in the nucleus of the four haploid spore progeny that result after sporulation. Moreover, this mutant allele leads to catastrophic developmental defects. I demonstrate that inhibiting Hsp90 activity, either genetically or pharmacologically, leads to impaired sporulation in yeast, highlighting the importance of the Hsp90 molecular chaperone in this developmental program.

By using fluorescent microscopic techniques to study the localization of different chaperones and co-chaperones, in Chapter 5 I was able to link Hsp104, Hsp70 and Hsp26

to both P-bodies and stress granules involved in mRNA metabolism. This is the first study to demonstrate chaperone localization to these dynamic centers of mRNA degradation, storage and recycling. I show that the three chaperones co-localize with both types of RNA granules, albeit with different localization dynamics. I discovered that upon stress the chaperones localize preferentially to stress granules or a combination of stress granule and processing body. Co-localization with both bodies simultaneously is exciting since the assembly, disassembly, and transition between the two compartments might prove to be important control points in mRNA metabolism being mediated by chaperones. Furthermore, I show that stress granule formation, but not formation of P-bodies is dependent on chaperone function, as ablation of Hsp104, Ssa1 and/or Hsp26 lead to a significant decrease in stress granules. Finally, I demonstrate that recruitment of chaperones to processing bodies is dependent on Hsp104, as cells lacking *HSP104* failed to recruit either Hsp70 or Hsp26 to processing bodies.

Taken together, the results presented in this dissertation establish novel roles for two distinct sets of molecular chaperone families. By taking advantage of fluorescent microscopy technology, I have linked the Hsp90 nuclear accumulation during quiescence to both chaperone function and development in yeast. Further, I link the Hsp100 family of chaperones and co-chaperones with a new role in mRNA granule formation. The studies presented in this dissertation will provide a basic framework for future studies into the importance of subcellular localization of molecular chaperones in diverse cellular processes.

Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

Strains and Growth Conditions

Yeast strains used in these studies are listed in Table 2-1. SWY519 and SWY3562 strains were a kind gift from Kathryn J. Ryan (Texas A&M University) (64). JJ816 and JJ817 strains were a kind gift from Jill L. Johnson (University of Idaho) (65). Diploid strain D818 was created by mating JJ817 and JJ816 and was confirmed by mating type testing. Strains were grown in selective (synthetic complete, SC) or nonselective (YP, 2% peptone, 1% yeast extract) media containing 2% glucose. Sporulation was induced by first growing cells in presporulation plates consisting of 3% Difco nutrient broth, 5% glucose, 1% yeast extract on patches (3 cm^2) that had been grown on YPD for 1 day. Cells were transferred to fresh presporulation plates for an additional day at 30°C. Cells were then shifted to supplemented liquid sporulation medium (0.01% zinc acetate, 1% potassium acetate, 1× histidine supplement, 1× uracil supplement, 1× leucine supplement), incubated at room temperature with aeration for 2 days followed by 2 days with aeration at 30°C. Pharmacological inhibition of Hsp90 in spore cultures was obtained by adding macbecin in dimethylsulfoxide (DMSO) or DMSO alone to concentrations ranging from 0-25 μM, with DMSO matching the total volume in each case before placing cells into supplemented liquid sporulation medium. Sporulation efficiency was quantified by determining the number of four-spore mature tetrads as a percentage of the total population of cells. For stationary/quiescent phase cultures, cells were grown for 3-4 days at 30°C in desired media to allow cells to reach quiescence. Starvation media was made using standard synthetic complete (SC) media (Sunrise Scientific, CA) lacking glucose, PO_4, NH_4, or both PO_4 and NH_4. Spent media was

obtained by growing cells in glucose rich media (YPD) for 3 days, quiescent cells were then removed by centrifugation.

Plasmid Construction

Plasmids used in these studies are listed in Table 2-2. pHsp82-GFP was built using recombinant cloning. Briefly, p414GPD-6XHis-Hsp82 was digested with *SacII* and *BamHI* and was used for gap repair via homologous recombination with PCR products amplified from the template plasmid pFA6a-GFP(S65T)-HIS3MX6, with ends homologous to the gapped plasmid (66, 67). pSba1-mCherry was constructed using recombinant cloning. p416GPD (*URA3*) was digested with *Xho*I and *Spe*I and used for gap repair. Sba1 was amplified from genomic DNA incorporating a flanking *Spe*I site and homology to the gapped plasmid as well as homology to mCherry. mCherry was amplified from the original *E. coli* codon-optimized mCherry expression vector incorporating a flanking *XhoI* site and homology to the 3' end of *SBA1* and the gapped plasmid (68, 69). Plasmids pTCA-Hsp82, pTCA-Hsp82/G313N and pTCA-Hsp82/E431K were a kind gift from Avrom Caplan (City College of New York) (70). pTCA-Hsp82-yEm-RFP, pTCA-Hsp82/G313N-yEm-RFP and pTCA-Hsp82/E431K-yEm-RFP fluorescent plasmids were constructed using recombinant cloning. Original plasmids were used for gap repair by digesting with *SacII*. yEmRFP was amplified from pRS316-Gap-Cherry (kind gift from Neta Dean, Stony Brook University), incorporating homology to Hsp90 and to the gapped plasmid as well as a flanking *XhoI* site (71). Colonies were confirmed by Western blot analysis and visually screened for the acquired purple fluorescence visible at the colony level. Plasmid p416Gal-v-Src has been previously described (72).

Plasmids pDcp2-RFP and pDual fluorescence (pRP1768) were a kind gift from Roy Parker (University of Arizona) (73). pHsp104-GFP and pHsp26-GFP were constructed using recombinant cloning. Briefly, Hsp104-GFP and Hsp26-GFP were amplified from genomic DNA (Open Biosystems strains gHsp104-GFP, gHsp26-GFP) incorporating a *XhoI* site and homology to the TEF1 promoter at the 5' end and a *SpeI* site and homology to the CYC1 terminator at the 3' end. p416TEF was digested with *SpeI* and *XhoI* and used for gap repair via homologous recombination with the pooled PCR products. Plasmid pSsa1-GFP was constructed using standard PCR and restriction enzyme cloning techniques using *XhoI* and *SpeI*.

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblot analysis were performed as described (74). Anti-Hsp82 was used at 1:2000 dilution (kindly provided by Avrom Caplan, City College of New York). Anti-GFP was used at 1:2000 dilution (Molecular Probes, OR).

HSP82-GFP Complementation Assay

A plasmid pHsp82-GFP or the empty vector p416GPD were transformed into the strain 5CG2 (*MATα ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1 hsc82::URA3 hsp82::GAL1-HSP82::LEU2*), which carries deletions of both *HSC82* and *HSP82*, and survives due to a *GAL1*-controlled copy of *HSP82* when grown on galactose-containing medium (75). Transformants were spotted and grown on selective plates containing galactose or glucose for 3 days and photographed.

v-Src Activity Assay

Cells were prepared for Western blot analysis by growing strains containing plasmid pGal-v-Src overnight in media containing glucose at 23°C. Early log phase cultures were collected and washed. Cultures at an initial density of 0.4 A_{600} (10 ml) were grown incubated for 10 hours in media containing galactose, to induce v-Src activity. Protein extracts were prepared using an altered alkaline lysis protocol to maintain tyrosine phosphorylation (76). Briefly, cells were collected and resuspended in 150 μl of 1% β-mercaptoethanol/1.85 M NaOH and incubated on ice for 10 minutes. 75 μl of 100% TCA was added, cells were incubated on ice for an additional 5 minutes. TCA precipitates were harvested by centrifugation at 5,000 x *g* for 2 min. The precipitate was washed once with 1 ml of acetone, resuspended in 2X SDS loading buffer supplemented with 0.1M Tris base. Samples were heated at 65°C for 10 min and subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose, incubated overnight with anti phosphotyrosine antibody (1 μg/ml, Millipore, MA) in TBS containing 3% nonfat dry milk followed by incubation with anti-mouse HRP conjugated secondary followed by detection by enhanced chemiluminescence (ECL) after incubation. The same blot was used to obtain load controls with an antibody against GPD, (anti-glucose-6-phosphate dehydrogenase, (Sigma, MO)).

Quiescent Phase Microscopy

Cells were collected by centrifugation, washed with water, and resuspended in 70% ethanol for 1 min. Cells were washed once more and collected by centrifugation and resuspended in water for visualization. DNA dye Hoechst 33342 was used to stain the nuclei of living cells for 5 minutes at a concentration of 10 μg/ml. Cells were observed using an epifluorescence microscope (Olympus BX60) equipped with a 100X immersion

oil objective and GFP, RFP and DAPI filter cubes. A minimum of 200 individual cells were analyzed for all experiments, chosen from distinct microscopic fields. Localization frequencies were calculated as a percentage of total (i.e. nuclear vs cytoplasmic), with representative images shown throughout. Images were captured with a Photometrics CoolSnap-fx cooled CCD camera driven by QED image-capturing software (Media Cybernetics, Bethesda, MD). Adobe Photoshop CS (San Jose, CA) was used to process all images.

Ethanol and Glucose Analysis

To determine glucose and ethanol concentrations, samples were taken at indicated time points during growth. Ethanol concentrations were measured using the EnzyChrom ethanol assay kit according to the protocol recommended by the manufacturer (BioAssay Systems, CA). Glucose concentrations were measured using a glucose assay kit following the protocol recommended by the manufacturer (Sigma, MO). Measurements were all done in duplicate.

Polymerase Chain Reaction Random Mutagenesis and Mutation Analysis

Random PCR mutagenesis was utilized to generate novel non-localizing alleles of *HSP82*. The *HSP82* gene was amplified from plasmid pHsp82-GFP using Taq polymerase in a reaction spiked with different levels of dNTPs (1mM dCTP and dTTP and 0.5mM dGTP and dATP) and 0.1 mM $MnCl_2$ to allow errors to occur during amplification. Homologous recombination was used to generate mutant plasmids. Briefly, pHsp82-GFP was digested with *SacII* and *BamHI* and used for gap repair with the mutagenic PCR products. Recombination plasmids were selected by transformation

into BY4741 cells on SC-His plates. Approximately 1,500 colonies were visually screened for lack of nuclear accumulation in quiescent phase.

Spore Viability Stress Assay

Sporulated cultures of either wild type *HSP82-GFP* or *hsp82-I578F-GFP* were exposed to 55°C heat shock via incubation in 0.2 ml tubes placed in a thermal cycler for the indicated duration and plated on YPD in triplicate. Cells were allowed to grow for 2 days at 30°C.

Meiotic Spore Microscopy

Ethanol fixation of sporulated cultures of cells bearing the *hsp82-I578F* mutant was found to severely disrupt nuclei, precluding analysis. Instead, vital nuclear staining of sporulating cultures was achieved by adding DAPI at 10 μg/ml into the liquid sporulation media. Meiotic divisions were followed until multiple nuclei were visible in wild type and *hsp82-I578F* cells.

Transmission Electron Microscopy

TEM analysis was performed essentially as described previously (77) with modifications. Instead of using agar-embedded aggregates for selection of the yeast for electron microscopy, homogenous aliquots of yeast cultures were processed as suspensions. As needed during infiltration in epoxy resin, low speed centrifugation (~ 1,500 *g*) was used to recover and concentrate cells. Following infiltration in low viscosity Spurr's resin, excess resin was removed from each of the specimen sediments, which were then transferred into Beem capsules and covered with appropriate aliquots of fresh resin which

was subsequently polymerized at 60°C. Following thin-sectioning on a diamond knife, the stained specimens were transferred to copper grids and viewed using a Jeol CM12 electron microscope operated at 80KV.

Processing Body Induction and Microscopy

P body experiments were performed using yeast cultures grown to mid-logarithmic phase and induced with the indicated stress. Briefly, cells were grown in the appropriate SC media in the presence of glucose to OD_{600} 0.3-0.5. Cells were then harvested by centrifugation, washed briefly in the inducing media (+/- glucose, 1M KCl) and centrifuged for 30 seconds. Cells were then resuspended in the inducing media incubated at 30°C with shaking for ten minutes, and then harvested by brief centrifugation, washed in induction media, briefly centrifuged and resuspended for microscopic assessment. Analysis of three different individual experiments was done to quantitate the data sets. . Quantitation of P-body and stress granule foci in the different background strains was done in a blind manner for three independent data sets totaling 250-350 cells.

All images were taken using an Olympus IX81 automated inverted microscope. Images were captured using a Hamamatsu ORCA-R2 high-resolution camera, with a Photonics Photoablation system (tunable from 375-900 nm). Images were taken using Z-series of 12-18 images of 0.2 μm and collapsed during analysis by Slidebook 5.0 by creating maximum projection images over cells Z-axis. Z-series projection stacks were deconvolved using the constrained iterative method with SlideBook 5.0 software package for 2-D, 3-D and 4-D imaging with multi-algorithm digital deconvolution.

Table 2-1. Yeast strains used in these studies.

Strain	Description	Reference
BY4741	Mat**a** *his3Δleu2Δmet15Δura3Δ*	Open Biosystems
SWY519	Matα *ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ade2-1::ADE2:ura3-1*	(64)
SWY3562	Matα *kap95-E126K trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 ade2-1::ADE2:ura3*	(64)
JJ816	Mat**a** *hsc82::LEU2 hsp82::LEU2 ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 met2 lys2* Yep24-*HSP82*	(65)
JJ817	Mat**α** *hsc82::LEU2 hsp82::LEU2 ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 met2 lys2* Yep24-*HSP82*	(65)
D818	Mat**a**/Matα *hsc82::LEU2 hsp82::LEU2 ade2-1/ade2-1 trp1-1/trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 met2/met2 lys2/lys2* Yep24-*HSP82*	This study
hsp104Δ	Mat**a** *hsp104::kan^r,his3Δleu2Δmet15Δura3Δ*	Open Biosystems
ssa1Δ	Mat**a** *ssa1::kan^r,his3Δleu2Δmet15Δura3Δ*	Open Biosystems
hsp26Δ	Mat**a** *hsp26::kan^r,his3Δleu2Δmet15Δura3Δ*	Open Biosystems

Table 2-2. Plasmids used in these studies.

Plasmid	Description
pHis6-Hsp82	p414-GPD-6XHis-Hsp82
pHsp82-GFP	p414-GPD-6XHis-Hsp82-GFP(S65T)-His3MX6
pSba1-mCherry	p416-GPD-Sba1-mCherry
pNL1	pHsp82-GFP mutagenic PCR isolate
pNL2	pHsp82-GFP mutagenic PCR isolate
pHsp82-I578F	pHsp82-GFP with single amino acid substitution at residue 578 (I- F) HSP82 driven from the GPD promoter, CEN/TRP1 vector
pTCA/Hsp82	HSP82 driven from the GPD promoter, CEN/TRP1 vector
pTCA/Hsp82-G313N	As pTCA/Hsp82, with G313N substitution
pTCA/Hsp82-E431K	As pTCA/Hsp82, with E431K substitution
pTCA/Hsp82-yEmRFP	Hsp82 tagged with y-EmRFP protein at C terminus
pTCA/Hsp82-G313N-yEmRFP	Hsp82-G313N tagged with y-EmRFP protein at C terminus
pTCA/Hsp82-E431K-yEmRFP	Hsp82-E431K tagged with y-EmRFP protein at C terminus
pHsp104-GFP	p413-TEF-Hsp104-GFP
pSsa1-GFP	p413-GPD-Ssa1-GFP
pHsp26-GFP	p413-TEF-Hsp26-GFP
pDcp2-RFP	p416-Dcp2-mRFP
pPab1-yEmRFP	p416-Pab1 tagged with yEmRFP
pDual fluorescence (pRP1768)	Edc3-mCh; Pab1-CFP; cen; URA marker

Chapter 3: Hsp90 nuclear accumulation during quiescence

NOTE: This chapter is derived from work that has, for the most part, been published in 2010: "Hsp90 nuclear accumulation in quiescence is linked to chaperone function and spore development in yeast." Molecular Biology of the Cell 21 63-72 (78). I am the primary author on this paper and was responsible for preparing the original manuscript. I performed all experiments described in these chapters. The publisher of MBoC, the American Society of Cell Biology, grants authors the right to revise, adapt, prepare derivative works, present, or distribute the manuscript provided that all such distribution is for noncommercial benefit and there appears always the ASCB copyright credit and link to the original publication of the manuscript in MBoC Online.

Chapter 3: Hsp90 nuclear accumulation during quiescence

Introduction

Cells respond to heat shock and a variety of different stresses by inducing a specific set of genes that allow them to deal with and ultimately recover from stress. Heat shock proteins (HSPs) function as molecular chaperones by binding unfolded proteins, preventing aggregation and ultimately facilitating their refolding. One of the most studied and best-described classes of chaperones is the Hsp90 family. Hsp90 is an essential protein chaperone in eukaryotes that is required for the activation of many regulatory and signaling client proteins (13). In *Saccharomyces cerevisiae*, two homologs of Hsp90 exist, *HSP82* and *HSC82 (5)*. *HSP82* is expressed at low levels in unstressed cells but is highly induced during stress. *HSC82* is constitutively expressed at an abundant level, yet is only induced 2-3 fold during stress (5). Deletion of either Hsp90 gene leads to no detectable phenotype, but double deletion of both is lethal at all temperatures (28). Hsp90 is generally not involved in nascent protein folding but rather has a dedicated set of protein clients consisting of signal transduction proteins such as steroid receptors, signaling kinases and transcription factors (79). Proper function of Hsp90 requires formation of a multichaperone complex including a variety of other chaperones and co-chaperones that regulate substrate transfer from the Hsp70 chaperone system (Figure 1-1). Subsequent maturation of both nuclear and cytoplasmic clients is regulated by Hsp90s intrinsic ATPase activity.

Hsp90 is one of the most abundant proteins in the cytosol of eukaryotic cells, and its abundance is increased further by stress (28). Hsp90 is abundant in the cytosol of all eukaryotes, whereas Hsp90 orthologs such as TRAP-1 and Grp94 are restricted to the mitochondrial matrix and endoplasmic reticulum, respectively (36, 37). Hsp90 is known to shuttle between the cytoplasm and the nucleus when in complex with immature steroid receptor substrates (e.g. glucocorticoid receptor, androgen receptor). Hsp90, alongside its late stage co-chaperones (TPR-domain immunophilins, FKBP52) and the client protein, shift localization from being predominantly in the cytoplasm, shifting to the nucleus upon activation by their distinct activating compounds (e.g. glucocorticoid, testosterone) (38). Once in the nucleus Hsp90 and its client protein disassociate (38). In *S. cerevisiae*, Hsp90 exhibits uniform localization throughout the cytoplasm and nucleus during vegetative growth, consistent with involvement in both cytoplasmic and nuclear processes (39). It is unknown whether this distribution is altered under conditions of cellular stress.

S. cerevisiae cells growing in rich media obtain their energy from the fermentation of glucose to non-fermentable carbon compounds, most notably ethanol (80). During this phase, termed logarithmic growth, cultures multiply rapidly until glucose is exhausted from the medium. Once glucose is depleted, cells enter the diauxic shift, where the culture ceases rapid growth and the cells undergo metabolic remodeling to utilize the non-fermentable carbon sources present in the medium via oxidative phosphorylation (80). After the diauxic shift, cells undergo very few doublings until the non-fermentable carbon sources have been depleted, and then ultimately cease to proliferate (80). At this point, the culture is in stationary phase, and most of the cells are

quiescent, a period of reduced metabolic activity similar to non-mitotic, or quiescent metazoan cells.

Yeast cells acquire a variety of characteristics defining quiescent phase, including cessation of proliferation, accumulation of storage carbohydrates such as glycogen and trehalose, increased stress resistance, thickening of the cell wall, and most importantly, the ability to survive extended periods of time without nutrients (81, 82). We have recently discovered that Hsp90 accumulates in the nucleus of quiescent cells. Despite the large body of knowledge surrounding the chaperone activity and binding partners of Hsp90, little is known about the subcellular localization of the protein during "normal" or stress conditions. We have recently discovered that Hsp90 accumulates in the nucleus of quiescent cells. The work described in this dissertation seeks to define the molecular role for the accumulation of Hsp90 in the nucleus of quiescent cells.

Results

Quiescent phase nuclear accumulation of Hsp90

To better understand the function of Hsp90, I examined the localization of Hsp90 using a strain with green fluorescent protein (GFP) inserted at the carboxy terminal end of the *HSP82* genomic locus, creating an Hsp82-GFP fusion protein (39). During logarithmic phase in rich medium at 30°C, Hsp82-GFP was uniformly distributed throughout the cell (Figure 3-1A). After two days of continued incubation, a distinct concentration was observed; Hsp82-GFP demonstrated nuclear localization as determined by coincident localization with Hoechst 33342 staining of DNA in live cells. The culture had by this time entered stationary phase, as characterized by the accumulation of large unbudded cells and cessation of proliferation as determined by optical density measurements. It was possible that the nuclear localization observed was due to a GFP-containing proteolytic fragment and not the entire Hsp82-GFP chimeric protein. Western blot analysis verified that the Hsp82-GFP chimeric protein remains intact during growth (Figure 3-1B). It was also possible that nuclear localization of Hsp82 did not reflect normal physiological activity but was an artifact caused by the addition of the GFP moiety. To address this possibility I asked whether Hsp82-GFP could act as the sole copy of Hsp90 in the cell. A plasmid bearing an Hsp82-GFP fusion was constructed using recombinant cloning. pHsp82-GFP was transformed into a strain with only one functional copy of Hsp90 under the galactose-inducible GAL1 promoter (*hsc82::URA3, hsp82*::Gal1-HSP82). This strain is only viable when cells are grown in galactose media. Upon transforming this strain with

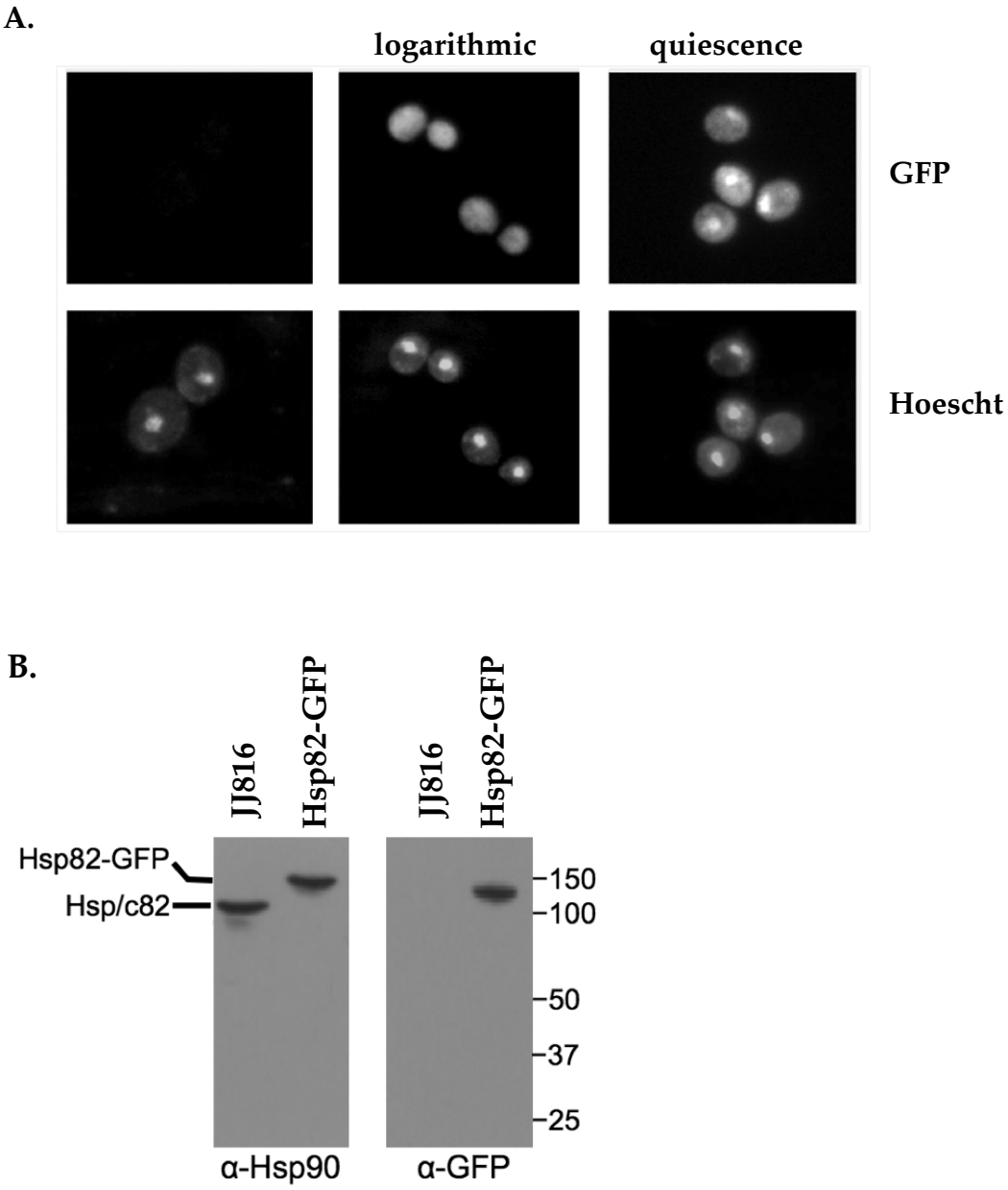
Figure 3-1. Hsp90 accumulates in the nucleus of quiescent cells.

A. Hps82-GFP (a genomic fusion) was examined during logarithmic phase and after 3 days of growth, when cells had entered quiescence. Photomicrographs are representative images of GFP localization and the nuclear positions as assessed by staining with Hoechst 33342 (78).

B. Strain JJ816, expressing the endogenous HSP82 or expressing HSP82-GFP after transformation with pHsp82-GFP and counter selection with 5-FOA were grown to late log phase and protein extracts were analyzed by immunoblot analysis. The GFP tagged protein is the only band reacting with anti-Hsp90 and anti-GFP antibodies, indicating that the fusion protein is not subject to significant levels of proteolysis in vivo (78).

C. Strain 5CG2 was transformed with plasmids pHsp82-GFP or the same corresponding empty vector p416GFD and plated on minimal SC-URA plates containing 2% glucose or 2% galactose as the sole carbon source and allowed to grow for 3 days (78).

Figure 3-1. Hsp90 accumulates in the nucleus of quiescent cells.



pHsp82-GFP, cells were able to maintain normal growth in the absence of both endogenous Hsp90 genes (Figure 3-1C). I next examined the localization of known Hsp90 co-chaperones (Sba1, Cdc37, Cpr7, Ssa1, Ydj1, Sti1, Hsp104, Hsp26 and Sse1/2) from the commercially available GFP collection during both logarithmic and quiescent phase (39). Similar to both Hsp90 isoforms (Hsp82 and Hsc82), I found that Sba1, the yeast p23 ortholog, and Ydj1, the Hsp70 ATPase activating co-chaperone, displayed quiescent phase nuclear accumulation (Figure 3-2A). Ydj1 was previously reported to localize to the nuclear and perinuclear space, consequence of a post-translational farnesylation event at the proteins carboxy terminal that anchors a sub-population in the contiguous ER/nuclear membrane (83). The majority of the chaperones examined (Sti1, Sse1/2, Cpr7 and Cdc37) remained distributed uniformly throughout the cytoplasm regardless of growth phase (Figure 3-2B). A third pattern of localization into small dynamic puncta was observed with Hsp104, Hsp26 and Hsp70 (Ssa1), which will be reported in Chapter 5 of this dissertation. These findings demonstrate that Hsp90, along with the co-chaperone Sba1, relocates to the nucleus after extended growth, exclusive of other known soluble Hsp90 partner proteins.

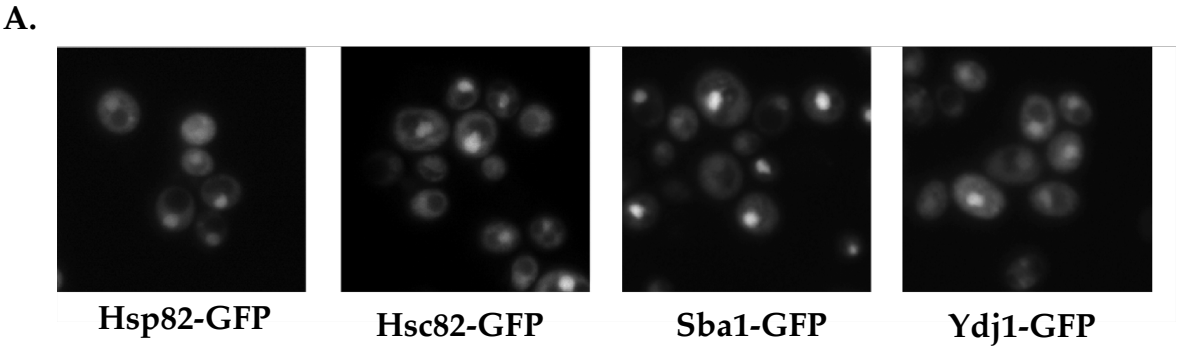
I next examined whether the nuclear accumulation of Hsp82 was a specialized consequence of entry into a quiescent state or a general response to cellular stressors. To differentiate between these two possibilities, a series of different stresses were applied to cells, including heat shock and toxic ethanol concentrations. I then examined the localization of Hsp82-GFP and observed that in contrast to quiescent cells, neither heat shock (42°C, 10 min) nor 10% ethanol resulted in nuclear accumulation (Figure 3-3A).

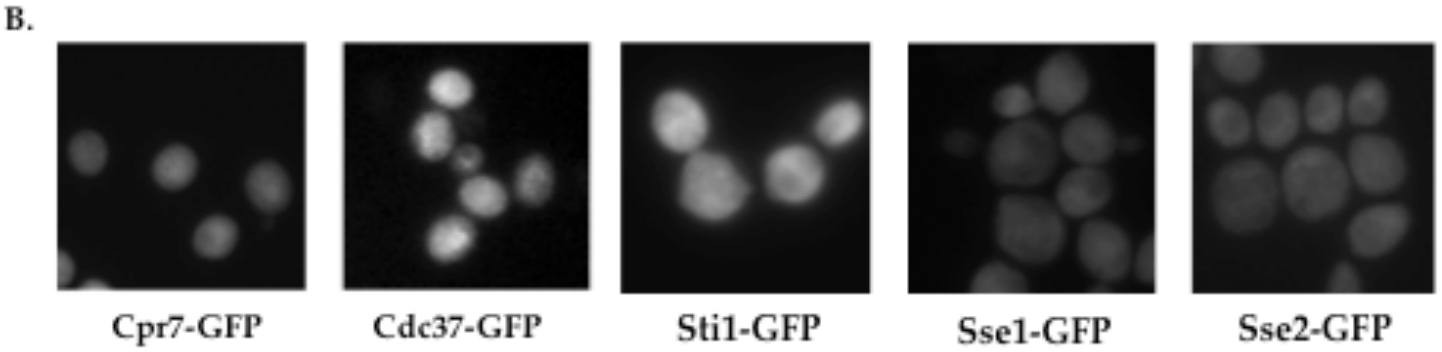
Figure 3-2. Nuclear accumulation of Hsp90 and co-chaperones.

A. Strains with genomic fusions of Hsp82-GFP, Hsc82-GFP, Sba1-GFP and Ydj1-GFP were grown for 3 days of rich media examined after entrance into quiescence. All four strains demonstrate nuclear accumulation. Only quiescent phase photomicrographs are shown (78).

B. Strains with genomic fusion of Cdc37-GFP, Cpr7-GFP, Sti1-GFP and Sse1/2-GFP were localized as described in A. Co-chaperones remained distributed uniformly throughout the cytoplasm (78).

Figure 3-2. Nuclear accumulation of Hsp90 and co-chaperones.





These results support the interpretation that both Hsp90 and Sba1 translocate to the nucleus specifically in response to the cells nutritional status.

To test whether nuclear translocation was occurring in response to depletion of a specific nutrient, cells containing Hsp82-GFP were grown to logarithmic phase and shifted to minimal media lacking different essential nutrients. Cells grown in the absence of glucose, a nitrogen source ($-NH_4$), phosphate ($-PO_4$) or lacking both nitrogen and phosphate demonstrated no nuclear translocation, demonstrating that acute withdrawal of an essential nutrient is not sufficient to lead Hsp82-GFP to translocate to the nucleus (Figure 3-3B). Furthermore, it was possible that a secondary metabolite was accumulating in the media during growth and progression through the diauxic shift that could induce the nuclear translocation of Hsp82. To test this possibility, I allowed a culture of cells bearing Hsp82-GFP to reach quiescence under normal growth conditions and collected the "spent" medium after centrifugation. Logarithmic phase Hsp82-GFP cells were resuspended in the "spent" medium and likewise failed to exhibit accumulation of the chaperone into the nucleus (Figure 3-3B).

I next analyzed the kinetics of Hsp90 nuclear translocation during growth in closer detail. Cells were grown and allowed to reach quiescence, demonstrating Hsp82-GFP nuclear accumulation (Figure 3-4, time 0). These cells were diluted into fresh medium and allowed to resume vegetative growth under normal conditions. Following three hours after the shift to glucose-rich media, Hsp82-GFP was observed to redistribute uniformly throughout the cell (Figure 3-4, representative images are shown from each sample).

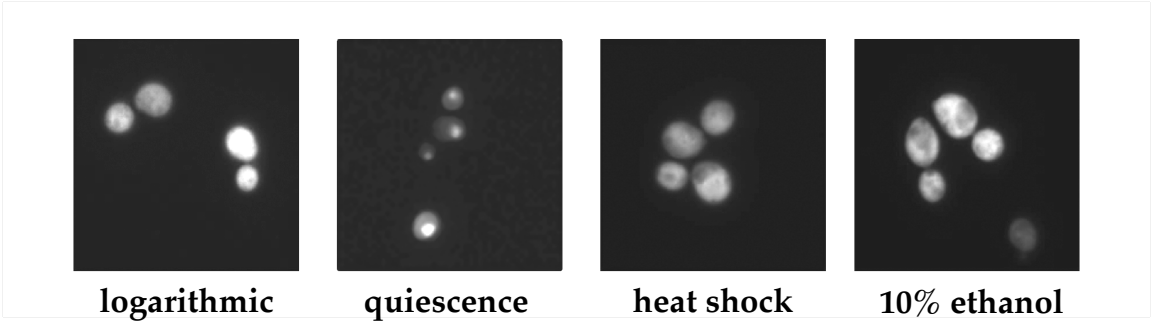
Figure 3-3. Hsp90 nuclear translocation is not a general stress response but specific to nutrient depletion.

A. Cells bearing the genomic fusion Hsp82-GFP were treated with 10% ethanol stress for 10 min or heat shocked for 1 hour at 42°C (78).

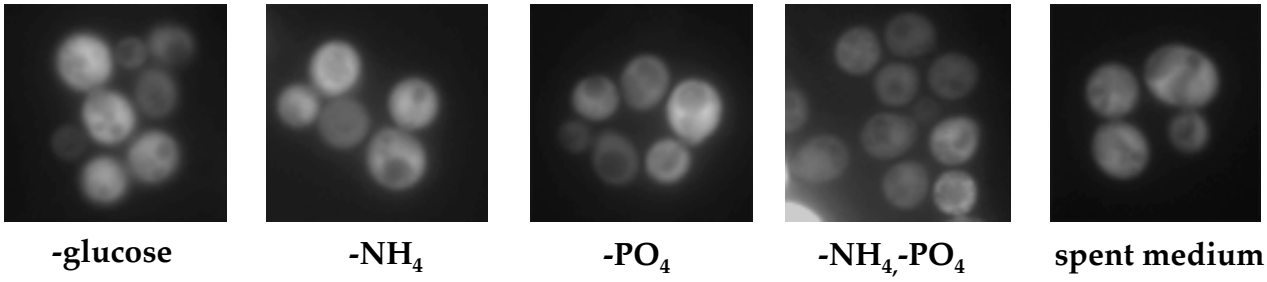
B. Logarithmic phase cells were transferred to synthetic medium lacking different nutrients, or spent medium that was collected from a stationary phase culture, images were taken 2 hours after incubation in nutrient deplete media (78).

Figure 3-3. Hsp90 nuclear translocation is not a general stress response but specific to nutrient depletion.

A.



B.



Ethanol and glucose concentrations were examined as cells were grown over a 24-hour period. After approximately 18 hours of growth, glucose was nearly exhausted from the media as determined by enzymatic assay, while as expected, ethanol produced during the initial fermentation had reached its maximum concentration. Concurrent with this, Hsp82-GFP was once again observed to translocate and accumulate in the nucleus. Hsp90 therefore seems to respond to events that are connected to glucose depletion and not the complete exhaustion of a carbon source since ethanol was still abundant (0.5%) at 18 hours. Taken together, these results suggest that the Hsp90 chaperone in yeast cells translocates to the nucleus in response to progression through the diauxic shift into quiescence. However, the precise signaling events that are required to initiate the change in subcellular localization are likely more complex as acute depletion of essential growth-limiting nutrients was unable to elicit the same response.

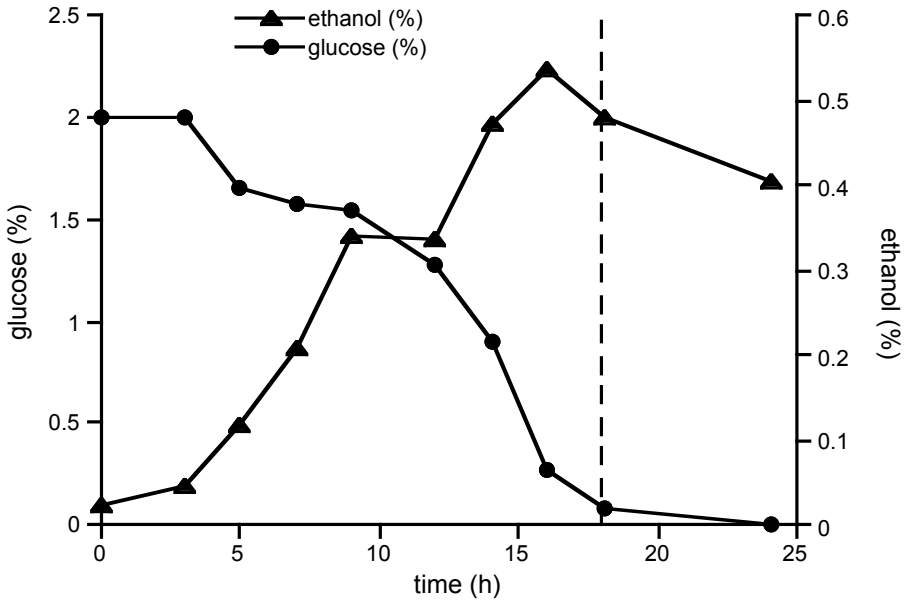
Hsp90 nuclear translocation is independent of Sba1 and requires the α/β importin system

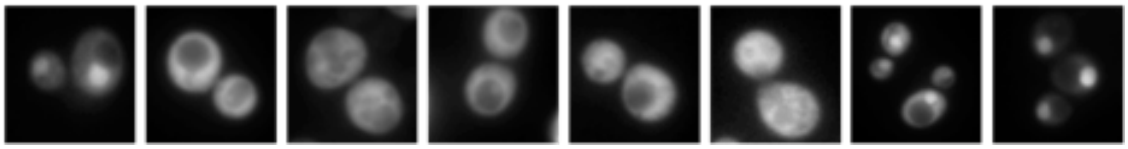
Sba1/p23 is a late-acting co-chaperone in the Hsp90 chaperoning cycle. Sba1 binds to the amino terminal nucleotide binding domains of both Hsp90 molecules in its functional dimer state and stabilizes the chaperone in the ATP-bound state (33, 84). The possibility then exists that nuclear accumulation of these two chaperones may be dependent on one another. Alternatively, Sba1 translocation to the nucleus during quiescence may

Figure 3-4. Hsp90 nuclear accumulation is a consequence of glucose depletion.

Quiescent cells bearing the Hsp82-GFP fusion were transferred to fresh YPD growth medium, the culture was examined for a growth period of 24 hours. Samples were collected at indicated time points processed for determination of ethanol (triangles), glucose (circles) or photomicroscopy as described in the *Materials and Methods* (78).

Figure 3-4. Hsp90 nuclear accumulation is a consequence of glucose depletion.





Time (h): 0 3 9 12 14 16 18 24

be regulated in parallel but independently of Hsp90. In order to distinguish and better understand these two possible models, we constructed a fusion of Sba1 with the dsRed variant mCherry on a plasmid. The plasmid-borne fusion of Sba1-mCherry acted in the same manner as was observed with the original GFP tagged fusion. Sba1-mCherry was distributed uniformly throughout the cell during logarithmic growth and translocated and accumulated in the nucleus during quiescence (Fig 3-5A). Co-localization in the nucleus was evident upon co-expression of both fluorescently tagged chaperones (Figure 3-5B). To directly examine whether Hsp90s nuclear accumulation required Sba1, we transformed BY4741 wild type strain and an isogenic *sba1*Δ strain with the plasmid-borne copy of pHsp82-GFP. In the absence of Sba1, Hsp90 translocated and accumulated in the nucleus in a manner nearly indistinguishable from quiescent wild type cells (Figure 3-5C). These data suggest that Sba1 translocates into the nucleus as a consequence of its association with Hsp90 as well as through an Hsp90-independent mechanism.

Proteins larger than 60 kDa require the assistance of a number of soluble factors to assist in the transport into and out of the nucleus through nuclear pore complexes. These factors, referred to as karyopherins, recognize nuclear localization signals (NLS) within the substrates that are required for nuclear targeting (85). Different karyopherins recognize proteins containing distinct nuclear export signals (NES) that are targeted for egress from the nucleus (85). To further dissect the molecular mechanisms that contribute to the nuclear accumulation of Hsp90 during quiescence, we transformed a plasmid-borne copy of fluorescently labeled Hsp82 (pHsp82-GFP) into known non-essential

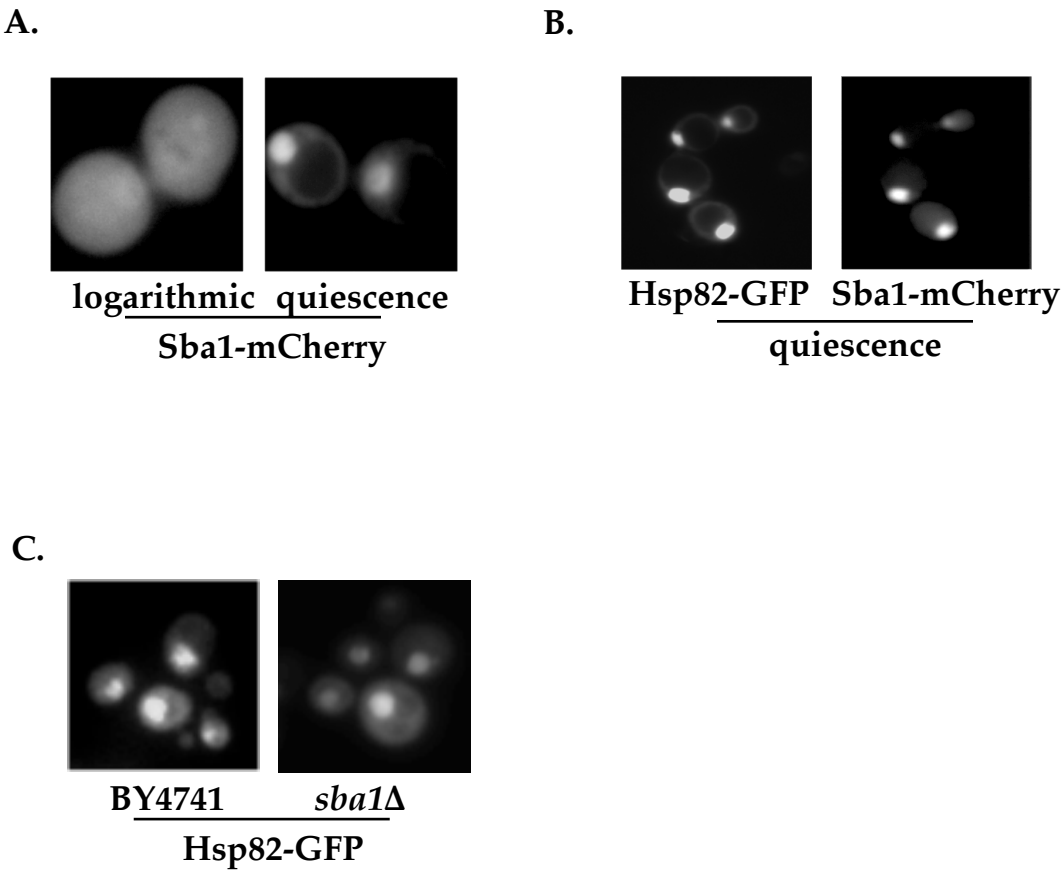
Figure 3-5. Hsp90 and Sba1 colocalize to the nucleus during quiescence.

A. BY4741 (wild type) was transformed with plasmid pSba1-mCherry and analyzed for subcellular localization (78).

B. BY4741 was transformed with both pHsp82-GFP and Sba1-mCherry was examined after entrance into quiescence. Control strains were transformed with only one of the chaperone fusions to confirm a lack of signal bleedthrough to other channels (78).

C. BY4741 and *sba1*Δ cells were transformed with plasmid pHsp82-GFP and examined upon entrance into quiescence as described in the *Materials and Methods* (78).

Figure 3-5. Hsp90 and Sba1 colocalize to the nucleus during quiescence.

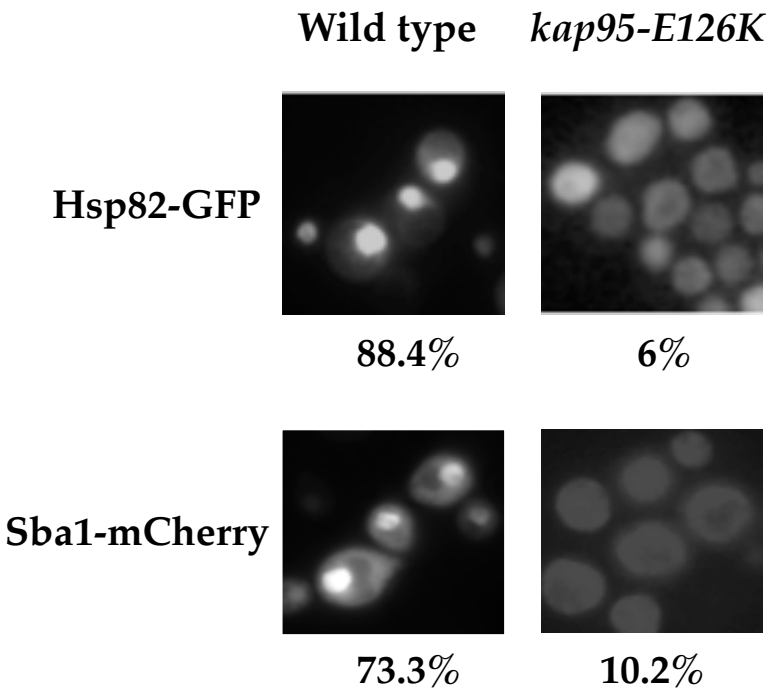


karyopherin gene deletions and examined the localization of Hsp82-GFP during both logarithmic growth and quiescence. Individual karyopherins have been identified as being responsible to be the sole protein required for the transport of substrates into and out of the nucleus. For example, Ssa4, an Hsp70 chaperone, accumulates in the nucleus of stressed cells, Kap142 is responsible for the nuclear export of this chaperone once conditions become favorable for the cell (62). Sba1-mCherry demonstrated similar results, with none of the different karyopherin deletions having any effect on its subcellular localization. *SRP1/KAP95* genes in yeast encode for the essential ortholog of the classical α/β importin system (86, 87). We acquired a strain bearing a temperature sensitive allele of *KAP95* (*kap95-E126K*) to examine whether Hsp90 and Sba1 are translocated into the nucleus by the classical α/β importin system. *kap95-E126K* cells displayed impaired nuclear accumulation of both chaperones, Hsp82-GFP and Sba1. (Hsp82 - 6% nuclear accumulation in quiescence vs. 88.4% for isogenic wild type and Sba1-mCherry - 10.2% nuclear accumulation in quiescence vs. 73.3% for isogenic wild type under semi-permissive conditions, 30˚C; Figure 3.6). Taken together, these data suggest that both Hsp90 and Sba1 accumulate in the nucleus of quiescent cells via the classical α/β importin system, *SRP1/KAP95*, and that the nuclear localization of Hsp90 does not require Sba1.

Figure 3-6. Hsp90 and Sba1 localize to the nucleus during quiescence via the *SRP1/KAP95* importin system.

Temperature sensitive strain *kap95-E126K* was transformed with either pHsp82-GFP or pSba1-mCherry allowed to reach quiescence at the semipermissive temperature of 30°C and analyzed as previously described. Numbers describe the percentage of cells demonstrating nuclear accumulation (78).

Figure 3-6. Hsp90 and Sba1 localize to the nucleus during quiescence via the *SRP1/KAP95* importin system.



Reduced Hsp90 nuclear accumulation is coincident with growth

To better understand the phenotypic relevance of Hsp90 nuclear accumulation during quiescence, I wanted to generate a non-localizing allele of *HSP82*. A plasmid-borne copy of Hsp82 was subjected to error-prone PCR in order to generate a *HSP82* non-localizing allele. Homologous recombination was used to generate the mutant plasmids which where then transformed into wild type yeast. Approximately 1,500 independent recombinants were screened for a lack of nuclear accumulation during quiescence. Two distinct non-localizing (NL) mutants were isolated and subsequently determined to be able to confer viability when present as the sole source of Hsp90 in the cell (Figure 3-7A,B).

The complete amino acid sequences for the two surviving NL mutants were acquired and analyzed. Both mutant alleles possessed numerous amino acid substitutions that were distributed throughout the entire length of the protein, lacking any common substitutions between them. NL1, the mutant allele with the least number of mutations, was chose to parse the substitutions and determine if any of the single point mutations was capable of conferring the non-accumulating phenotype. Of the five amino acid changes that were initially found within the mutant NL1 allele, the substitution of isoleucine 578 to phenylalanine in the carboxy terminal domain of the protein was sufficient to block nuclear accumulation of Hsp90 upon glucose exhaustion (Figure 3-7C). This isoleucine residue is highly conserved within the fungal lineage as well as in humans, yet no obvious NLS could be distinguished in the surrounding regions. Since we were able to identify at least two distinct non-localizing alleles of *HSP82*, quiescent-phase nuclear accumulation of Hsp90 is apparently not required for survival. In spite of

this, a more detailed analysis of the growth characteristics of the *hsp82-I578F* non-accumulating mutant demonstrated a pronounced lag in the transition to logarithmic phase from resting in quiescence, reflecting a possible defect in the re-entry into the cell cycle from G_0, as well as an slower overall growth rate (Figure 3-7E). Furthermore, the identification of this mutant allele allowed us to ask whether Sba1 could to translocate to the nucleus independently of Hsp90. Figure 3-7D demonstrates that Hsp90 accumulation is reduced from 95.4% in wild type to 17.4% in the *hsp82-I578F* non-accumulating mutant allele. In addition, Sba1-mCherry nuclear accumulation was reduced from 85.6% to 38.9%, relative to the wild type Hsp82 to the *hsp82-I578F* mutant yet the localization is not completely eliminated. These data suggest that the nuclear accumulation of Sba1 in quiescence is both a consequence of its association with Hsp90 as well as an Hsp90-independent mechanism.

Figure 3-7. Generation and examination of nonlocalizing *HSP82* alleles.

A. Two novel *HSP82-GFP* mutant alleles were identified that failed to accumulate in the nucleus in quiescence, Hsp82-GFP^{NL1} and Hsp82-GFP^{NL2} . (78)

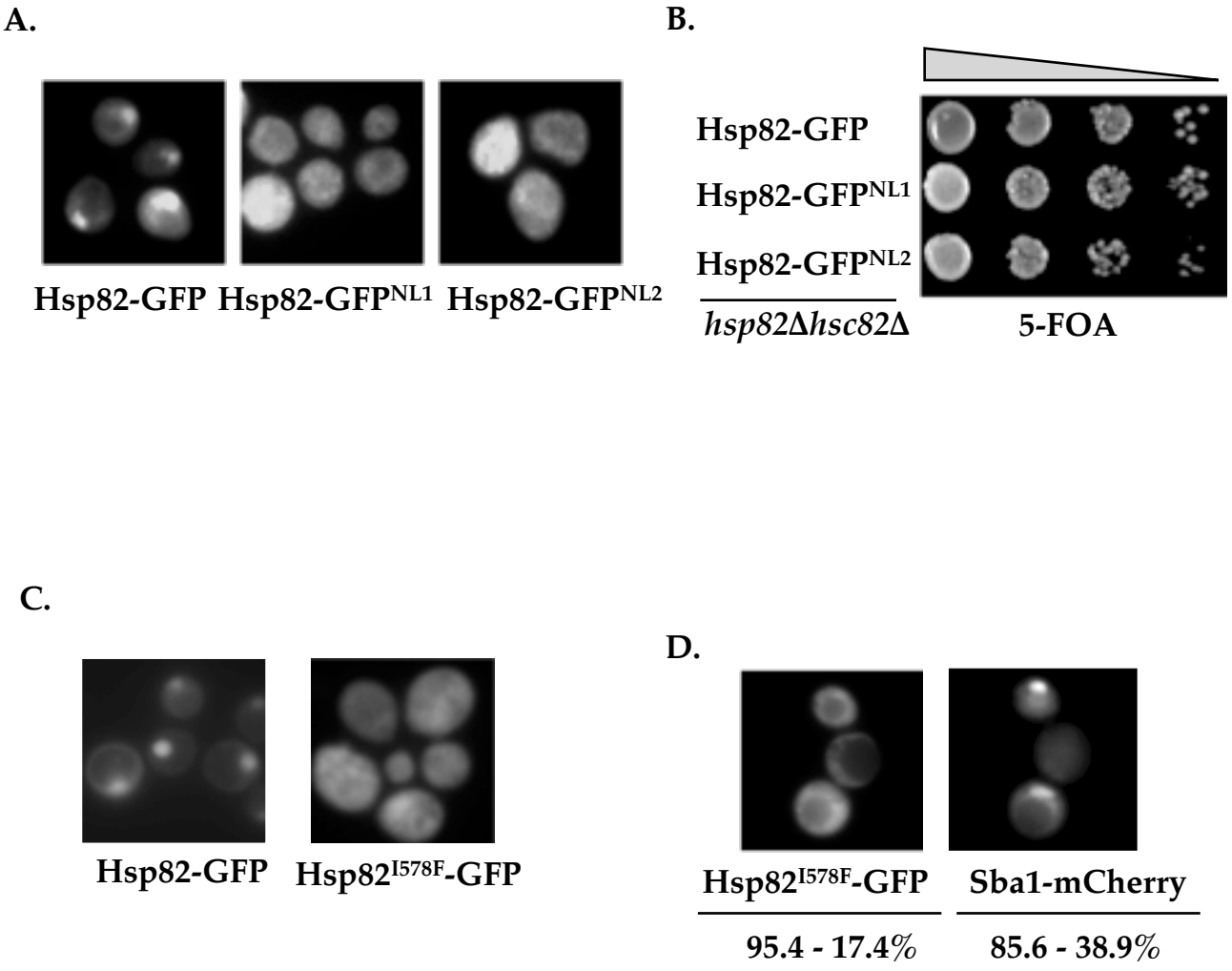
B. Mutant allele plasmids were transformed into strain JJ816 and serial dilutions were plated on 5-FOA to counter select for the loss of Yep24-HSP82 plasmid, resulting in the mutant NL alleles as the sole source of Hsp90 in the cell (78).

C. Mutant allele Hsp82-GFP^{NL1} was sequenced and of the five distinct amino acid substitutions detected, I578F was sufficient to confer the nonlocalizing phenotype (78).

D. JJ816 cells transformed with plasmid pHsp82-I578F as the only source of Hsp90 and pSba1-mCherry were grown to quiescence and localization for both chaperone fusions was analyzed. Numbers describe the percentage of cells demonstrating nuclear accumulation under wild type *HSP82-GFP* vs. the *hsp82-I578F* mutant (78).

E. JJ816 cells harboring either the *hsp82-I578F* mutant (closed triangles) or wild-type *HSP82-GFP* were diluted form a quiescent phase culture into rich media (YPD) and growth rates were followed by optical density (OD_{600}) for 50 hours (78).

Figure 3-7. Generation and examination of nonlocalizing *HSP82* alleles.



Discussion

In this chapter, I describe a new localization pattern for the Hsp90 chaperone based on the cell's nutrient availability. Using a microscopy based approach I observed that Hsp90 was found distributed uniformly throughout cytoplasm in logarithmic phase cells and accumulated in the nucleus of quiescent cells (Figure 3-1A). This observation demonstrates a starvation stress event that triggers the translocation of Hsp90 into a specialized subcellular compartment. In addition, this chapter describes the accumulation of Sba1, the yeast p23 ortholog, into the nucleus as a consequence of its association with Hsp90 as well as an Hsp90-independent mechanism (Figure 3-2A, 3-7D). These findings imply that a minimal Hsp90 chaperone system is necessary for one or more nuclear processes during long periods of relative metabolic inactivity. A set of other co-chaperones tested remained distributed throughout the cytoplasm regardless of growth phase (Figure 3-2B), establishing that not all Hsp90 associated co-chaperones are transported to the nucleus upon entrance into quiescence.

I have observed that the nuclear accumulation of Hsp90 within the nucleus is specific to glucose exhaustion and progression through the diauxic shift into quiescence, yet the precise signaling events that trigger this accumulation are likely complex, as acute depletion of specific nutrients did not elicit nuclear accumulation (Figure 3-3, 3-4). Entrance into quiescence is characterized not only by the depletion of nutrients but also by the regulation of a distinct set of signaling pathways (88). These pathways include the protein kinase A (PKA) and the TOR pathways, which are apparent negative regulators of the transition into quiescence, and the SNF1 pathway, which acts as a positive activator of this transition (88). Preliminary evidence has shown that overexpression of

Bcy1, a negative regulator of PKA, is able to promote Hsp90 nuclear accumulation during normal logarithmic growth, in the presence of high concentrations of glucose. The involvement of the PKA pathway in the accumulation of Hsp90 in the nucleus could be contributed to a mechanism such as phosphorylation of Hsp90 after glucose exhaustion. Hsp90 phosphorylation has been linked to its chaperoning function. Sch9, a serine/threonine kinase, was previously identified to regulate Hsp90 activity, as lack of Sch9 suppresses growth defects observed in a strain containing low levels of Hsp90 and its co-chaperones, as well as leading to the increased activation of Hsp90-dependent clients (89). Sch9 has been implicated in previous studies to share functional overlap with the catalytic subunits of PKA (90). However, a direct role for the role of Sch9 in the regulation of Hsp90 still remains elusive. Recently, it has been reported that Wee1, the only true tyrosine kinase in yeast, phosphorylates a conserved tyrosine residue in the N-domain of Hsp90 (91, 92). Hsp90 phosporylation by Wee1 is cell cycle regulated and affects Hsp90 ATPase activity and its ability to chaperone a selected group of clients, comprised primarily of protein kinases (91, 92). Wee1 is an interesting Hsp90 client protein to consider when examining the regulation of Hsp90 nuclear translocation. We are yet to define the nature of the signal that is leading to the accumulation of Hsp90.

Yeast *SRP1/KAP95* are an essential gene pair that encode the classical α/β importin system required for the translocation of non-diffusible protein through the nuclear pore complex into the nucleus (87). We identified Kap95 as the importin responsible for the nuclear translocation of Hsp90, as a temperature sensitive allele of *KAP95* lead to a substantial decrease in the nuclear accumulation of both Hsp90 and Sba1 at the permissive temperature of 30°C (Figure 3-6). Nuclear translocation may be

specific to the Kap95 importin, as nuclear accumulation was unaffected in an examination of non-essential importin deletion strains (see Results). Our observation is not the first time that Hsp90 has been observed to translocate to the nucleus. Avian Hsp90 is a predominantly cytoplasmic chaperone that can be in part relocated to the nucleus via overexpression of a client receptor protein (93). However, it seems unlikely that Hsp90 is "piggybacking" with a sole client protein into the nucleus given the extent of nuclear accumulation that we observe. Previous data suggest that distinct Hsp90 chaperone machines may form to aid in the trafficking of different client proteins; FKBP52, an Hsp90 co-chaperone, can alter the subcellular distribution of Hsp90 in mammalian cells. When a client protein, such as p53 or the glucocorticoid receptor are in complex with Hsp90, immunophilins such as FKBP52 link to the cell's cytoskeletal tracks to assist in the nuclear transport of the client protein (94). No ubiquitous consensus sequence has been fully described for nuclear translocation beyond the inclusion of hydrophobic or basic stretches of amino acids. I was unable to identify a clear nuclear localization sequence (NLS) or nuclear export sequence (NES) within *HSC82* or *HSP82* after examination of their amino acid sequences. The region surrounding the non-accumulating single amino acid substitution (I578F) is not consistent with either NLS or NES. In published crystal structures, I578 is obscured within the C-terminal dimerization domain of Hsp90. It is possible that this mutation might create a perturbation in Hsp90 tertiary structure leading to its failure in accumulating in the nucleus.

The data presented in this chapter are significant as they reveal a novel subcellular localization of an essential molecular chaperone, Hsp90 in yeast cells. I show that Hsp90

accumulates in the nucleus of quiescent cells. However, the lack of Hsp90 nuclear accumulation leads to a pronounced lag phase in the transition from quiescence to logarithmic growth as well as an overall slower growth rate, suggesting a possible defect in the re-entry into the cell cycle from quiescence. Hsp90 has recently been implicated to play an integral role in many human diseases, including cancer, neurodegenerative diseases, viral infections and fungal pathogenesis, a better understanding of the overall molecular mechanisms of Hsp90 will provide a strong foundation for developing targeted therapeutics against these different diseases.

Chapter 4: Hsp90 is required for proper spore development

NOTE: This chapter is derived from work that has, for the most part, been published in 2010: "Hsp90 nuclear accumulation in quiescence is linked to chaperone function and spore development in yeast." Molecular Biology of the Cell 21 63-72 (78). I am the primary author on this paper and was responsible for preparing the original manuscript. I performed all experiments described in these chapters. The publisher of MBoC, the American Society of Cell Biology, grants authors the right to revise, adapt, prepare derivative works, present, or distribute the manuscript provided that all such distribution is for noncommercial benefit and there appears always the ASCB copyright credit and link to the original publication of the manuscript in MBoC Online.

Chapter 4: Hsp90 is required for proper spore development

Introduction

The heat shock protein 90 (Hsp90) chaperone is a highly abundant, evolutionarily conserved molecular chaperone (79). Molecular chaperones, by definition, assist other proteins in folding and prevent the aggregation of unfolded proteins, which can be deleterious to the cell. The function of Hsp90 and other molecular chaperones is essential during normal vegetative growth, yet their importance is particularly noted during conditions that interfere with proper protein folding, such as high temperatures, starvation or other stresses (13). Consequently, Hsp90 as well as many other chaperones are highly stress inducible. Hsp90 is essential for the proper progression of many fundamental cellular processes (13).

Diploid cells of the yeast *Saccharomyces cerevisiae* alter their growth in response to the availability of distinct nutrients (88). In the presence of nutrients diploid cells divide by budding in a manner indistinguishable from haploid cells. In the presence of a poor nitrogen source, such as proline, diploid cells trigger a developmental cascade that results in a pseudohyphal form (88). When faced with extreme starvation conditions, like the absence of nitrogen and a carbon source, yeast diploid cells exit mitotic growth, undergo meiosis and ultimately sporulate (95). Once sporulation is complete, the spore wall will confer on the spore resistance to a variety of environmental stresses (95).

Synchronization of the events required for sporulation is a regulatory challenge. Sporulation in *S. cerevisiae* depends on the proper progression and completion of one event and the initiation of the next in order to develop a mature viable spore. Similar to

gamete formation in higher eukaryotes, sporulation in yeast is tightly regulated at the transcriptional level (96). The transcriptional program is divided into three temporal categories of genes; early, middle and late sporulation-specific genes (95). Early genes are required for meiotic chromosome dynamics such as genetic recombination and homolog pairing, middle genes function in meiotic divisions and the beginnings of spore wall morphogenesis and finally late genes are required for proper spore wall assembly leading to acquired stress resistance (95).

In the presence of a nonfermentable carbon source, diploid cells starved for nitrogen will undergo meiosis (95). Two rounds of meiotic divisions (Meiosis I, Meiosis II) results in chromosomes packaged as four haploid nuclei, which are encapsulated within a double membrane resulting in four autonomous prospores. The actual process of spore formation begins during meiosis II. The prospores mature into true spores through synthesis of a stress resistant spore wall. During spore formation, the four distinct layers of the spore wall are sequentially synthesized within the lumen of the prospore, ultimately surrounding the four meiotic products. The spore wall is composed of two inner spore wall layers, which resemble the vegetative cell wall (mannan layer and beta-glucan polysaccharide layer), and the two outer layers, which contain spore-specific materials (chitosan layer and dityrosine layer) (Figure 4-1, Cartoon inset). After completion of the spore wall, the anucleate mother cell will serve as an ascus to encapsulate the four spores of the tetrad (95).

In this chapter, I demonstrate that Hsp90 activity and localization are required for proper spore development in yeast. I show that Hsp90 localizes to the nucleus of all four spores. Using a mutant (I578F) that fails to localize to the nucleus upon glucose

exhaustion, we show that this mutant also fails to accumulate in the nucleus of the four haploid spore progeny that result after sporulation. Moreover, this mutant leads to catastrophic developmental defects. I demonstrate that inhibiting Hsp90 activity, either genetically or pharmacologically, leads to impaired sporulation in yeast, highlighting the importance of the Hsp90 molecular chaperone in this developmental program.

Results

Reduced Hsp90 nuclear accumulation is coincident with developmental defects

Simultaneous starvation of *S. cerevisiae* diploid cells for nitrogen and a fermentable carbon source leads to the activation of the sporulation program and meiosis (95). As a result, four haploid spore progeny are produced (95). If Hsp90 localizes to the mitotic nucleus during glucose starvation, as described in Chapter 3, I reasoned that Hsp90 should localize to the nuclei of the four haploid spore progeny possibly involving Hsp90 in the sporulation program of *S. cerevisiae*. Diploid strains were generated expressing either the non-localizing mutant *hsp82-I578F-GFP*, or wild type *HSP82-GFP* as the sole source of Hsp90. Cells were induced to initiate the meiotic program by growth in sporulation medium (see Materials and Methods). Hsp90 was indeed observed to localize to all four-spore nuclei (Figure 4-1A), consistent with my previous observations that Hsp90 nuclear localization is not a general stress response, but linked to nutrient status. A surprising phenotype was observed when I examined spore formation in cells bearing the *hsp82-I578F-GFP* non-localizing mutant; very few mature four-spore asci were observed. Instead, I observed a variety of sporulation defects ranging from atypically large cells apparently devoid of spores to cells with compartments indicative of immature (non-birefringent) disorganized spores (Figure 4-1A). A nearly complete failure to produce spores was observed after sporulation efficiency was quantified by scoring the acquisition of mature four-spore asci (Figure 4-1B). This defect is not likely a result of failure to initiate or proceed

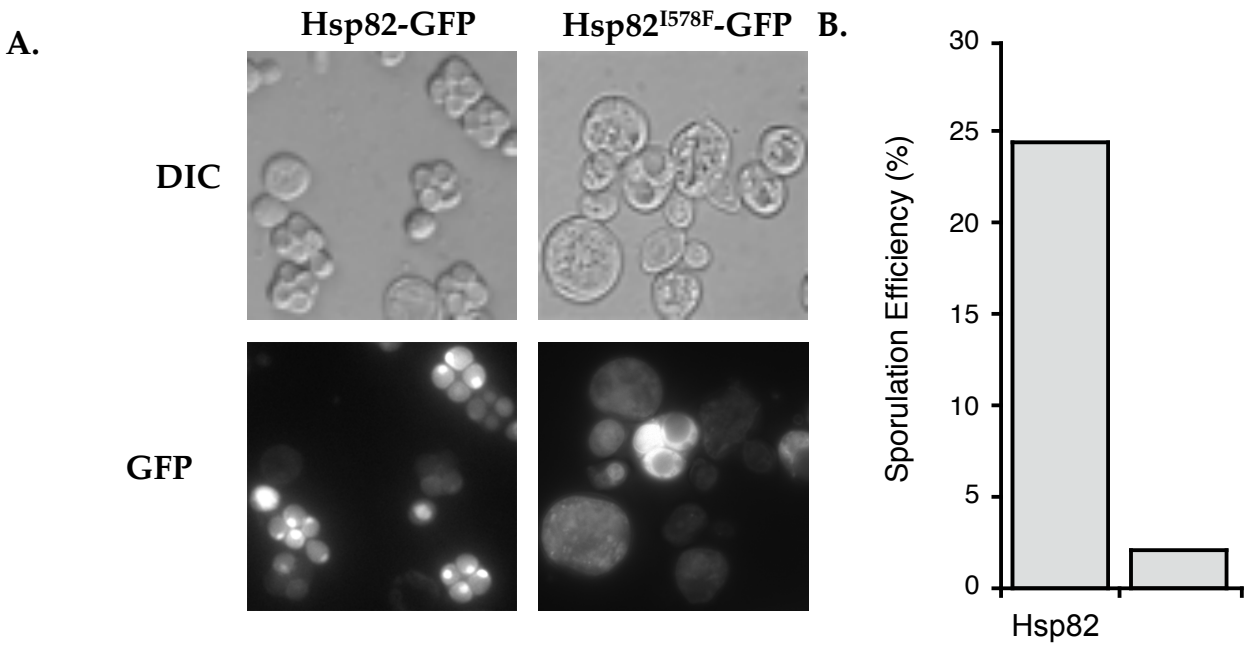
Figure 4-1. The *hsp82-I578F* mutant displays sporulation defects

A. D818 diploid cells carrying either pHsp82-GFP or pHsp82-I578F-GFP as the sole copy of Hsp90 were sporulated and visualized using differential interference contrast (Nomarksi; DIC) optics and epifluorescence (78).

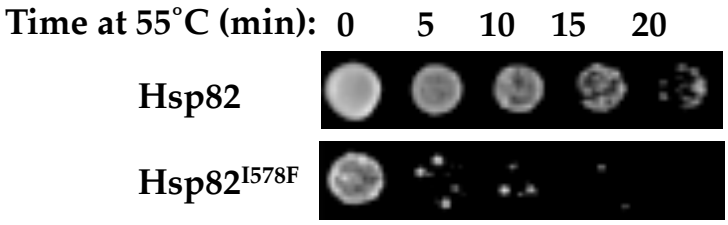
B. Quantitation of sporulation efficiency of cells from (A) (78).

C. Sporulated cultures of D818 bearing pHsp82-GFP or pHsp82-I578F-GFP were subjected to 55°C heat shock and plated for viability at 30°C as described in Materials and Methods (78).

Figure 4-1. The *hsp82-I578F* mutant displays sporulation defects



C.



through meiosis, as I was able to detect multiple disorganized nuclear bodies by Hoechst staining of the sporulated diploids expressing *hsp82-I578F-GFP* as the sole source of Hsp90 in the cell (Figure 4-2). However, I was unable to precisely quantitate meiotic progression in the *hsp82-I578F-GFP* mutant strain, as nuclei not encapsulated within the prospores appeared to be highly unstable.

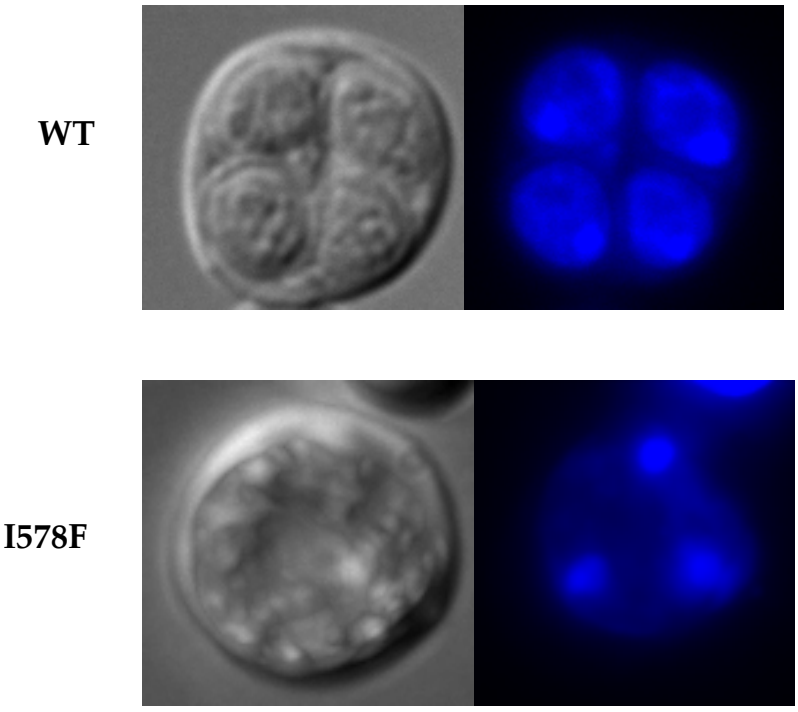
Sporulation in the yeast *S. cerevisiae* leads to the production of highly stress-resistant cells capable of withstanding severe environmental insults such as desiccation and heat shock (95). I subjected cultures of sporulated diploid cells expressing either *HSP82-GFP* or *hsp82-I578F-GFP* to a lethal 55˚C heat shock and plated for viability at 30˚C (Figure 4-1C). *HSP82-GFP* cells retained viability for up to 20 minutes of lethal heat shock while cultures expressing *hsp82-I578F-GFP* demonstrated drastically reduced levels of survival after only 5 minutes. These data are consistent with the reduced production of spores in this strain. These findings demonstrate that Hsp90 in addition to accumulating in the nucleus of cells transitioning into quiescence, diploid cells with a mutation conferring deficiencies in Hsp90 nuclear targeting exhibit drastic sporulation defects.

Similar to metazoan differentiation programs, sporulation is induced in specific cell types in response to nutrient stimulus, and is characterized by an ordered progression of morphogenic stages ultimately leading to a differentiated state (95). The spore wall is compromised of two outer spore layers containing spore-specific materials, chitosan and dityrosine, and two inner spore wall layers, similar to a vegetative cell (Figure 4-3A), (95). To investigate the morphological and structural defects associated with the loss of

Figure 4-2. Progression of meiosis in hsp82-I578F cells.

D818 diploid cells carrying either pHsp82-GFP or pHsp82-I578F-GFP as the sole copy of Hsp90 were sporulated in the presence of Hoechst 33342 stain as described (Materials and Methods) and photomicrographs obtained at approximately 20 h after induction of meiosis. Multiple disorganized nuclei are observed in these cells, indicating meiotic progression but lack of spore formation (78).

Figure 4-2. Progression of meiosis in hsp82-I578F cells.



Hsp90 nuclear accumulation, I sporulated *HSP82-GFP* and *hsp82-I578F-GFP* diploid cells and examined them by transmission electron microscopy. The ultrastructure of a typical wild type spore is illustrated by the ascus depicted in Figure 4-3A. Multiple defects were exhibited in the spore wall formation of *hsp82-I578F-GFP* asci. Assembly of the outer dityrosine layer is a possible defect associated with the failure of proper sporulation as exhibited in Figure 4-3B, where the spore wall seems to have ruptured, retaining an incomplete dityrosine ghost structure and releasing all of its contents. Asci with partial to a complete failure to assemble spores can be observed in Figure 4-3C and D. Localization of Hsp90 into the nucleus may therefore be required for one or more critical steps in progression through spore morphogenesis, including but not limited to spore wall synthesis.

Hsp90 activity and localization are intertwined and required for proper sporulation

The preceding data raise the possibility that functional Hsp90 is required within the nucleus prior to the initiation of sporulation to generate viable, intact spores. Alternatively, the *hsp82-I578F-GFP* mutant, in addition to blocking nuclear accumulation, could also be hypomorphic for Hsp90 activity, and loss of chaperoning function sufficient to produce the observed defects. I attempted to differentiate between these two scenarios by assaying the ability of *hsp82-I578F-GFP* to support the activation and function of the Hsp90 client tyrosine kinase v-Src in yeast cells (97). When expressed, heterologously under control of the GAL promoter, v-Src promiscuously phosphorylates a

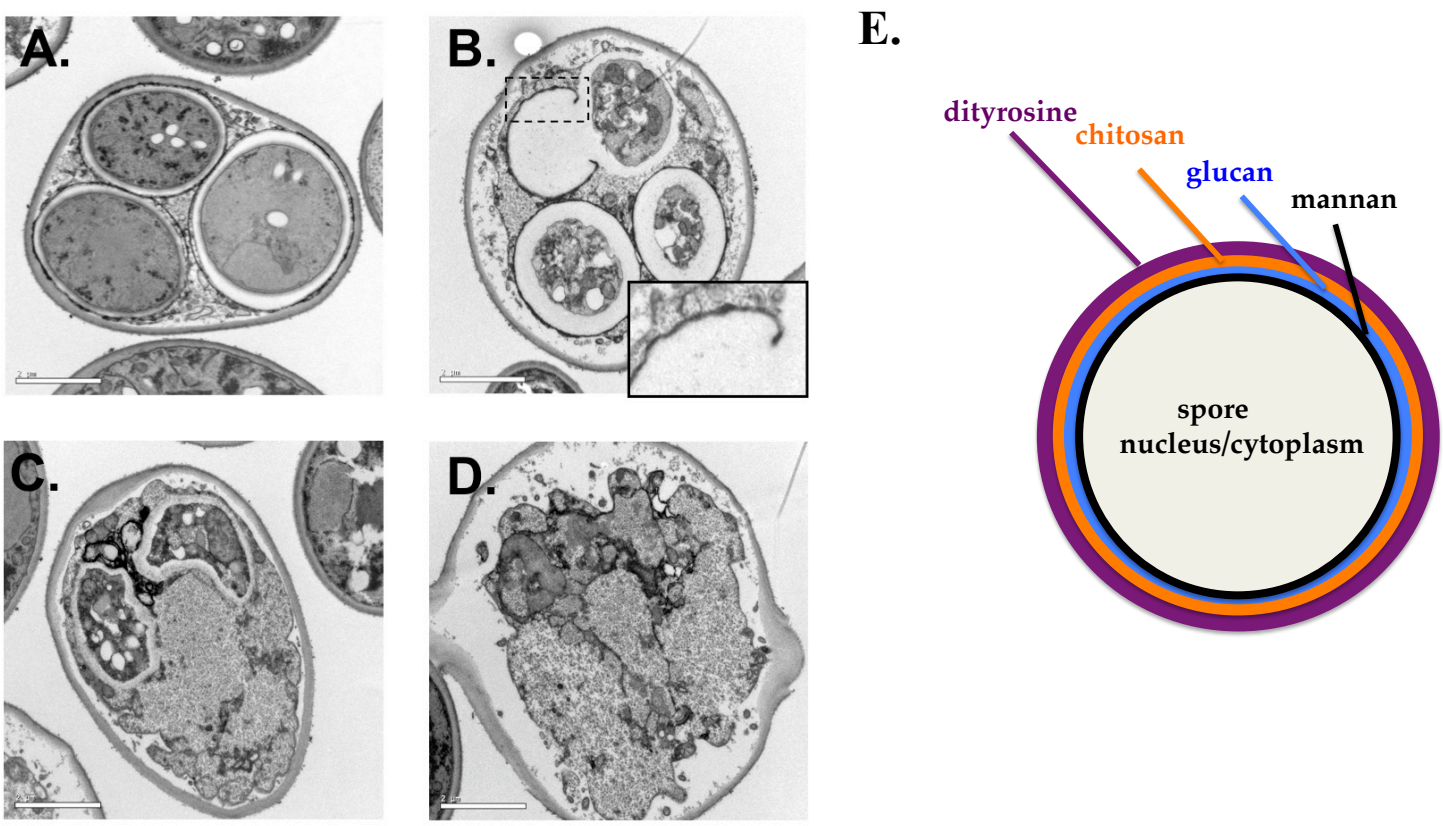
Figure 4-3. *hsp82-I578F* mutant leads to spore wall construction defects.

A. D818 cells carrying pHsp82-GFP as the sole copy of Hsp90 exhibit normal spore formation as shown by transmission electron microscopy (78).

B-D. D818 cells carrying pHsp82-I578F-GFP as the sole copy of Hsp90 exhibit diverse sporulation defects. Inset in **(B)** is a 2X digital zoom of dashed rectangle. Bar = 2 μm (78).

E. Cartoon depicting a wild type spore surrounded by its four layers, mannan, beta-glucan, chitosan and dityrosine.

Figure 4-3. *hsp82-I578F* mutant leads to spore wall construction defects.



number of endogenous yeast proteins in an Hsp90 dependent manner, which can be detected by immunoblot with anti-phosphotyrosine antibodies (72). When grown in galactose (Figure 4-4, Lane 2), the characteristic banding pattern representative of widespread tyrosine phosphorylation can be observed in cells bearing a wild type *HSP82* (Figure 4-4). When grown in glucose medium (Figure 4-4, Lane 1), no signal is observed confirming antibody specificity. *hsp82-I578F-GFP* cells showed a substantial reduction in tyrosine phosphorylation, indicative of a reduction in Hsp90 chaperoning activity. In order to place these results in context, I also examined the activity of two previously characterized *HSP82* mutant alleles, *hsp82-G313N* and *hsp82-E431K* (70). When compared directly to an isogenic wild type allele (see Material and Methods), the single amino acid substitution of G313N displayed a complete loss of v-Src activity, while the E431K mutant allele appeared similar to wild type (Figure 4-4). Previous functional analysis using steroid receptor clients demonstrated that the G313N substitution leads to severe diminution of Hsp90 chaperoning activity while E431K behaves like wild type under most conditions tested (70, 98). My results are consistent with these previous findings and demonstrate that our I578F substitution also leads to diminution of v-Src signaling. I next examined the ability of G313N and E431K mutant alleles to support sporulation when present as the sole source of Hsp90 in diploid cells. Notably, while diploid cells expressing *hsp82-E431K* or wild type HSP82 exhibited similar sporulation efficiencies, *hsp82-G313N* diploid cells displayed dramatic sporulation defects similar to those observed with the non-localizing *hsp82-I578F* mutant (Figure 4-5A,B). These data suggest

Figure 4-4. Non-localizing mutant I578F demonstrates vSrc-signaling deficiencies

D818 cells bearing plasmids pTCA-Hsp82, pTCA-Hsp82/G313N and pTCA-Hsp82/E431K or pHsp82-GFP or pHsp82-I578F-GFP and p416Gal-v-Src were induced by growth in the presence of galactose for 10 h (gal), or maintained in glucose (glc). Protein extracts were assayed by immunoblot for bulk tyrosine phosphorylation (α-p-tyr). Levels of glucose phosphate dehydrogenase were assessed as a loading control (α-GPD) (78).

Figure 4-4. Non-localizing mutant I578F demonstrates vSrc-signaling deficiencies

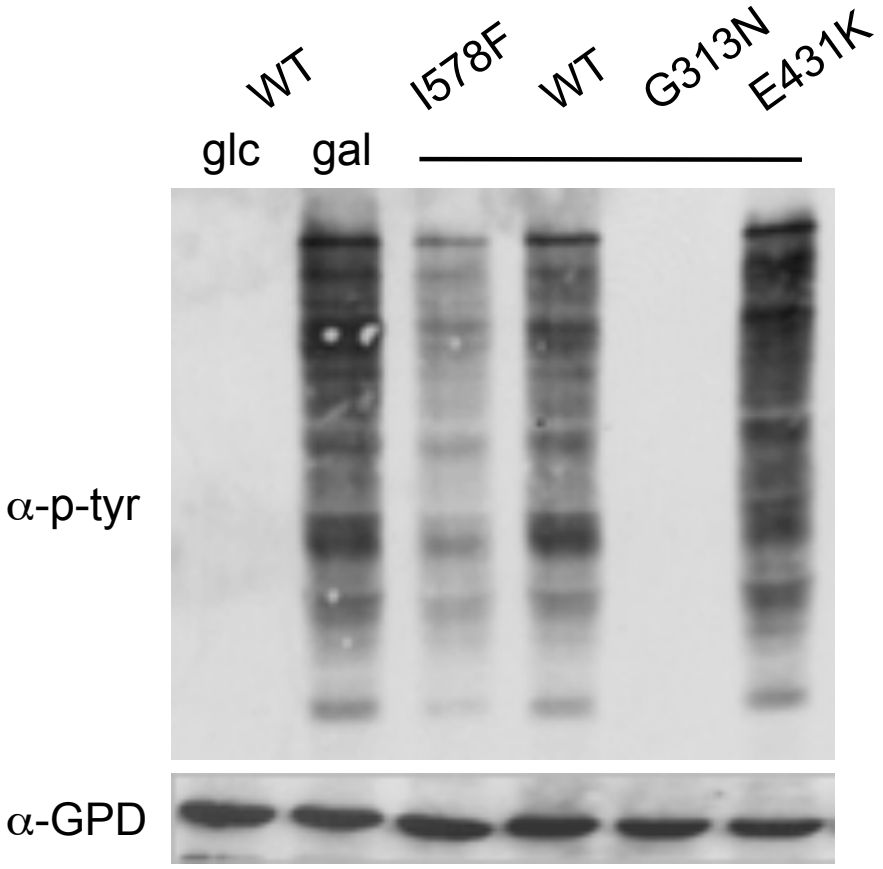


Figure 4-5. Reduction in Hsp90 activity is sufficient to cause defective sporulation

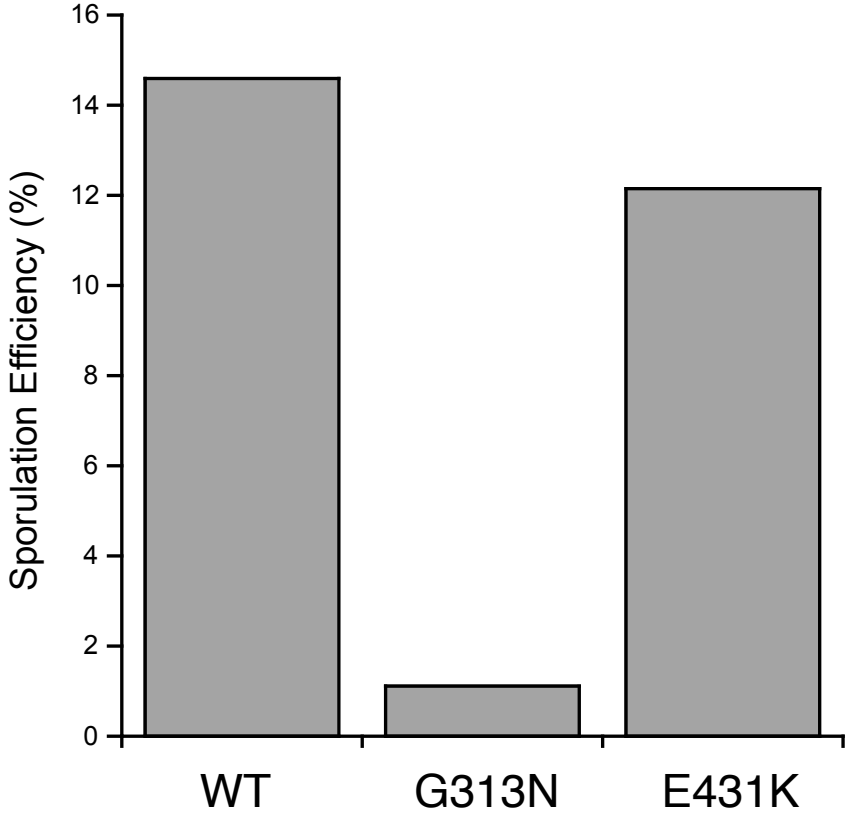
A. D818 diploid cells carrying pTCA-Hsp82, pTCA-Hsp82/G313N or pTCA-Hsp82/E431K as the sole copy of Hsp90 were sporulated and visualized using differential interference contrast (Nomarksi; DIC) optics.

B. D818 diploid cells carrying either pTCA-Hsp82, pTCA-Hsp82/G313N or pTCA-Hsp82/E431K as the sole copy of Hsp90 were sporulated and quantified (78).

Figure 4-5. Reduction in Hsp90 activity is sufficient to cause defective sporulation

A.

B.



that reduction in the chaperone activity of Hsp90 is sufficient to inhibit proper sporulation. To test this hypothesis further, I asked whether pharmacological inhibition of endogenous Hsp90 in wild type cells with macbecin, an Hsp90-specific inhibitor, would have the same effect on sporulation (99). Indeed, as shown in Figure 4-6A, macbecin treated cells resulted in a dose-dependent inhibition of sporulation efficiency, with sporulation completely blocked at established concentrations (~ 20 μM) known to inhibit Hsp90 in yeast cells (100).

We next investigated the localization status of the *hsp82-G313N* and *hsp82-E431K* proteins by constructing Hsp90-fluorescent protein chimeras using the recently described yeast-enhanced monomeric red fluorescent protein (yEm-RFP; (71)). I selected this approach in order to aid in microscopic detection since yEm-RFP is exceptionally bright, and the *hsp82-G313N* and *hsp82-E431K* mutants and their corresponding wild type parent are expressed from low copy plasmids. During logarithmic growth, wild type and the two *HSP82* mutant proteins were distributed uniformly throughout the entire cytoplasm. Strikingly, *hsp82-G313N-yEmRFP* failed to accumulate in the nucleus of quiescent cells, *hsp82-E431K-yEmRFP* also displayed reduced localization but to a much lesser extent (Figure 4-6B). These findings establish a convincing correlation between Hsp90s subcellular localization, and its functional status that suggests that these two characteristics may be functionally linked.

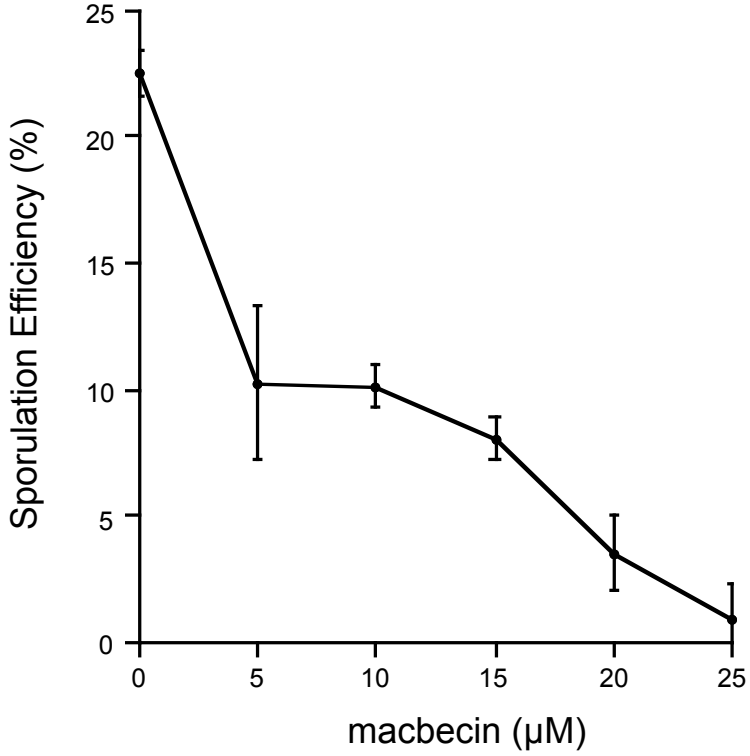
Figure 4.6. Hsp90 pharmacological inhibition and reduced nuclear accumulation of *HSP90* mutants

A. Wild type diploid cells were treated with the indicated concentrations of macbecin during sporulation and efficiency calculated as previously described. Error bars represent the standard deviation from two replicates (78).

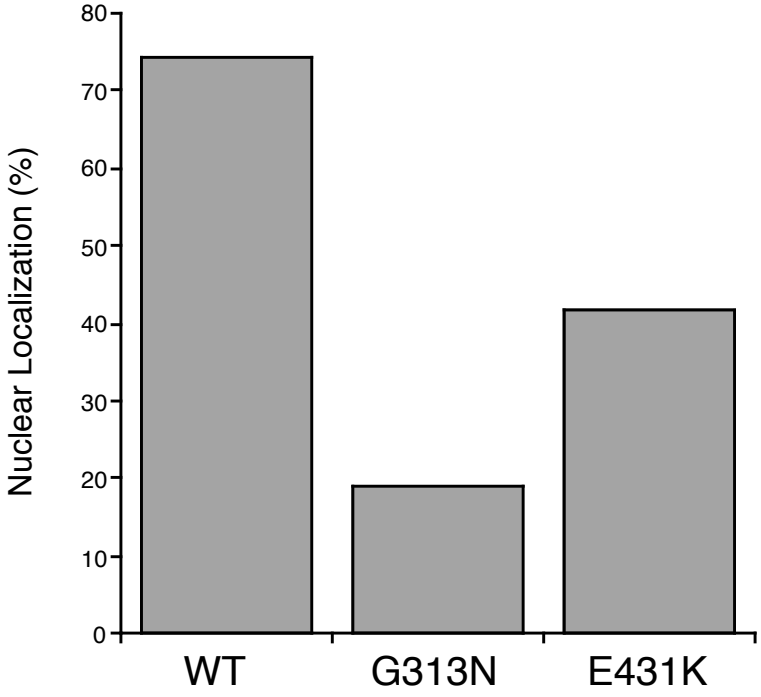
B. D818 cells bearing the pTCA-Hsp82-yEm-RFP, pTCA-Hsp82/G313N-yEm-RFP and pTCA-Hsp82/E431K-yEm-RFP plasmids as the sole copy of Hsp90 in the cell were grown to quiescent phase and subcellular distribution assessed (78).

Figure 4.6. Hsp90 pharmacological inhibition and reduced nuclear accumulation of *HSP90* mutants

A.



B.



Discussion

In this chapter, I describe a new role for the Hsp90 chaperone in development based on nutrient-responsive nuclear accumulation. In the last chapter, I described a single amino acid substitution, I578F, that blocks nuclear accumulation upon glucose exhaustion. If Hsp90 localizes to the mitotic nucleus during glucose starvation, as described in Chapter 3, I then reasoned that Hsp90 should localize to the nuclei of the four haploid spore progeny produced during sporulation. I observed that Hsp90 indeed localizes to all four spore nuclei (Figure 4-1A), consistent with our previous data that Hsp90 nuclear localization is a specific response to nutrient starvation, and not a general stress response. Surprisingly, sporulating cells expressing the *hsp82-I578F-GFP* non-localizing mutant, failed to produce mature spores. Instead, the mutant exhibited a variety of sporulation defects ranging from atypically large cells apparently devoid of spores to cells with compartments indicative of immature, disorganized spores (Figure 4-1A). As mentioned in the previous chapter, the I578F mutation might lead to a change in the tertiary or quaternary structure of Hsp90, preventing nuclear accumulation. This possibility is supported by our finding that the Hsp90-G313N mutant, and to a lesser extent Hsp90-E431K, likewise failed to localize to the nucleus during quiescence, linking Hsp90 functional status to its subcellular localization (Figure 4-6B). Therefore, at this time, we cannot completely divorce Hsp90 function from subcellular localization or truly conclude which is the deficiency responsible for the lack of proper development. However, it is interesting to note the convergence of localization and function phenotypes; in a search for mislocalzing *HSP82* mutants I uncovered a functionally impaired allele, and two mutants previously isolated on the basis of loss of function were

found by me to mislocalize. I attempted to force the cytoplasmic retention of wild type Hsp82 by incorporating tandem nuclear export sequences to Hsp82 (Hsp82-NES-GFP), yet these experiments were not successful due to extensive proteolysis of Hsp82-NES-GFP, suggesting that a formal test of the role of nuclear accumulation during quiescence will require further in-depth study.

The dramatic defects observed in spore formation and maturation in cells genetically (Figure 4-1, 4-5), or pharmacologically (Figure 4-6A) depleted of Hsp90 activity strongly implicates this protein chaperone in the process of yeast meiotic development, and suggests the existence of one or more formerly unappreciated client proteins involved in this important biological process. Upon initiation of sporulation, three distinct temporal categories of genes have been characterized; early, middle and late. Early genes are generally needed for meiotic chromosome dynamics such as recombination, middle genes are required for proper meiotic division as well as early steps in spore biogenesis, and late genes include genes required for spore wall assembly (95). Carefully regulated gene expression during sporulation is required to properly orchestrate these events. It is important to note that I did not observe defects in meiotic nuclear division, which suggests that Hsp90 plays a critical role downstream of meiosis and is likely important in proper spore wall synthesis (Figure 4-2). During normal vegetative growth, the growing yeast cell wall is comprised of beta-glucan and mannan, in contrast, the spore wall contains four layers; the inner two are found in normal vegetative cells (mannan and beta-glucan) and two spore wall specific layers, chitosan and dityrosine (Figure 4-3). Both chitosan and dityrosine layers confer much of the observed stress resistance characteristics common to spores (95). Proper spore wall

assembly in *S. cerevisiae* is dependent on the mitogen activated protein kinase (MAPK), Smk1 (101). Different Smk1 mutant alleles have been described to result in improper spore wall formation in a manner comparable to what we observe in our I578F mutant (101). Future work will be required to precisely determine the identity of the Hsp90 clients responsible for the panoply of defects I have documented during spore assembly when the protein chaperone is prevented to localize to either the pre-meiotic and spore nuclei.

Although we are the first to report the developmentally regulated subcellular targeting of the protein chaperone Hsp90 in yeast, there is precedence in higher eukaryotes. Hsp90 and different co-factors also play an important role in tissue differentiation and development in *Arabidopsis*; delayed chloroplast development is observed when the chloroplast-specific Hsp90 homolog is mutated (26). The Hsp90 homolog TRAP1 also exhibits differential localization throughout the various developmental stages of the *Dictyostelium* amoeba (102). Interestingly, during development of prespore cells, localization of TRAP1 to the prespore-specific vacuole is required for spore wall formation (102). In a similar manner, our results suggest a functional and perhaps spatial role for the Hsp90 chaperone in a developmental process in the brewing yeast *S. cerevisiae.*

Chapter 5: Chaperone requirements in mRNA processing body and stress granule formation

Chapter 5: Chaperone requirements in mRNA processing body and stress granule formation

Introduction

The yeast molecular chaperone Hsp104, along with its bacterial homolog ClpB, are members of the Hsp100 superfamily of AAA+ ATPases (ATPase associated with diverse cellular activities) (40). Unlike the Hsp90 family of chaperones, Hsp104 does not fold any specific set of 'client' proteins, but rather assists in reactivating stress-denatured proteins by solubilizing protein aggregates. Hsp104 is expressed at low levels under normal growth conditions but is highly induced upon stress (44). Hsp104, which has no ortholog in higher eukaryotes, forms hexameric ring structures that aid in solubilizing aggregated proteins [57]. Hsp104 has attracted much attention due to its role in thermotolerance, defined as the cells ability to withstand sudden lethal temperature shock (5). Cells can acquire thermotolerance to lethal heat stress when presented with a milder heat stress by inducing expression of heat shock proteins that will aid in restoring activity to heat inactivated proteins (46). Over-expression of Hsp104 alone is sufficient to allow cells to be thermotolerant without the preconditioning treatment (46). When Hsp104 is present, yeast cells are 100- to 1000-fold more thermotolerant than cells lacking Hsp104 (44). Although *HSP104* deletion strains demonstrate no observable phenotypes during normal growth conditions, when exposed to stresses such as heat, radiation and ethanol, they exhibit a marked decrease in survival (47).

Hsp104 functions together with the Hsp70 family of chaperones to refold non-native proteins trapped in aggregates (48, 49). Ssa1, Ssa2, Ssa3 and Ssa4 encode

chaperone proteins in the yeast *Saccharomyces cerevisiae* that belong to the SSA subfamily of cytosolic Hsp70 proteins (103). The HSP70 family of chaperones is a large family of proteins conserved from bacteria to humans that aid in binding newly translated proteins to assist in proper folding, as well as preventing the aggregation of folded proteins (104). To this extent, Hsp70 functions closely with Hsp104 to reactivate proteins that have aggregated after denaturing stresses. Hsp70 is thought to both stimulate the ATPase activity of Hsp104 as well as assist in the extraction of polypeptides from aggregates by presenting unstructured regions to Hsp104 (Figure 1-2) (46).

It was recently reported that a small heat shock protein (sHSP), Hsp26, aids in the disaggregation of aggregated proteins by Hsp104 (52). Hsp26 assembles with misfolded proteins and bolsters access of the disaggregation machinery, composed of Hsp104/Hsp70, to the aggregated proteins. Hsp26 is a sHSP that is highly induced as cells enter quiescence or when they are subjected to heat stress (53). *In vitro,* Hsp26 forms into oligomeric structures and assemble into large complexes with aggregation prone proteins when stressed with heat (54). Hsp26 along with other sHSPs, such as Hsp42, aid in the refolding of these aggregation-prone proteins by holding them in a semi-competent state, where Hsp104 can carry out reactivation of the denatured proteins, with assistance from the Hsp70 chaperone system (Figure 1-2) (52).

When heat shocked, yeast cells accumulate electron-dense anomalies that are likely aggregated proteins in both the cytoplasm and nucleus of the cell (63). These aggregates dissolve during recovery from heat shock but only when a functional Hsp104 is expressed (41). Furthermore, Hsp104 has been shown to accumulate in the periphery of aggregates both in the cytoplasm and nucleus of heat-stressed cells (63). Hsp104

subcellular localization has been extensively detailed; along with its localization around aggregates, Hsp104 accumulates in the nucleus of heat stressed cells using a karyopherin-independent mechanism (63). Additionally, messenger RNA (mRNA) splicing, an activity that is highly sensitive to stress, recovers more quickly when Hsp104 is present (105). I have recently discovered that Hsp104, Hsp70 and Hsp26 coalesce into discrete dynamic foci in the cytoplasm of quiescent cells. In this chapter I will describe this novel subcellular localization and demonstrate that more than being aggregated proteins, these foci represent both mRNA processing bodies (P-bodies) and stress granules.

P-bodies are RNA-protein granules found in eukaryotic cells that contain non-translating mRNAs and proteins involved in translational repression and mRNA degradation (106). P-body formation is induced by conditions such as glucose deprivation, hyperosmotic stress, high cell density, or when mRNA decapping is inhibited (107). P-bodies have been associated with mRNA storage, normal mRNA degradation, nonsense mediated (NMD) mRNA decay, miRNA-mediated repression and translational repression (107). In a similar manner, stress granules are formed when cells are stressed and bulk translation is inhibited (108). Stress granules are messenger ribonucleoprotein (mRNP) aggregates that contain mRNA and stalled translational preinitiation complexes that accumulate during stress. Stress granules are considered sites of mRNA triage where mRNP complexes are monitored and routed to reinitiation, degradation or storage (108). Both types RNA granules are common in size and number but are distinguished by the different proteins that accumulate within them. While P-bodies contain proteins involved mostly in mRNA decay (Dcp1/2, Lsm1-7, Edc3, Pat1) stress granules contain factors involved in translational reinitiation (eIF4GI, eIF4GII,

Pab1), while several protein components and mRNA species are shared between structures (Xrn1, eIF4E, Fas-activated serine/threonine phosphoprotein (FAST)) (73).

A close association between P-bodies and stress granules has been observed in cells and evidence exists for the exchange of components between these bodies. While stress granules have been primarily studied in mammalian cells, these dynamic aggregates of untranslating mRNAs in conjunction with translation initiation factors, have been shown to dock and undock from P-bodies in a dynamic matter (109). It has recently been shown in yeast that stress granule assembly is dependent on P-body formation, whereas P-body formation is independent of stress granule formation (110). These observations suggest that P bodies are sites for decisions on mRNA fate, either degrading mRNAs or transferring mRNAs to stress granules for reentry into translation (110). The purpose of the work described in this chapter is to define the molecular role for the accumulation of Hsp104, Ssa1 and Hsp26 into both P-bodies and stress granules.

Results

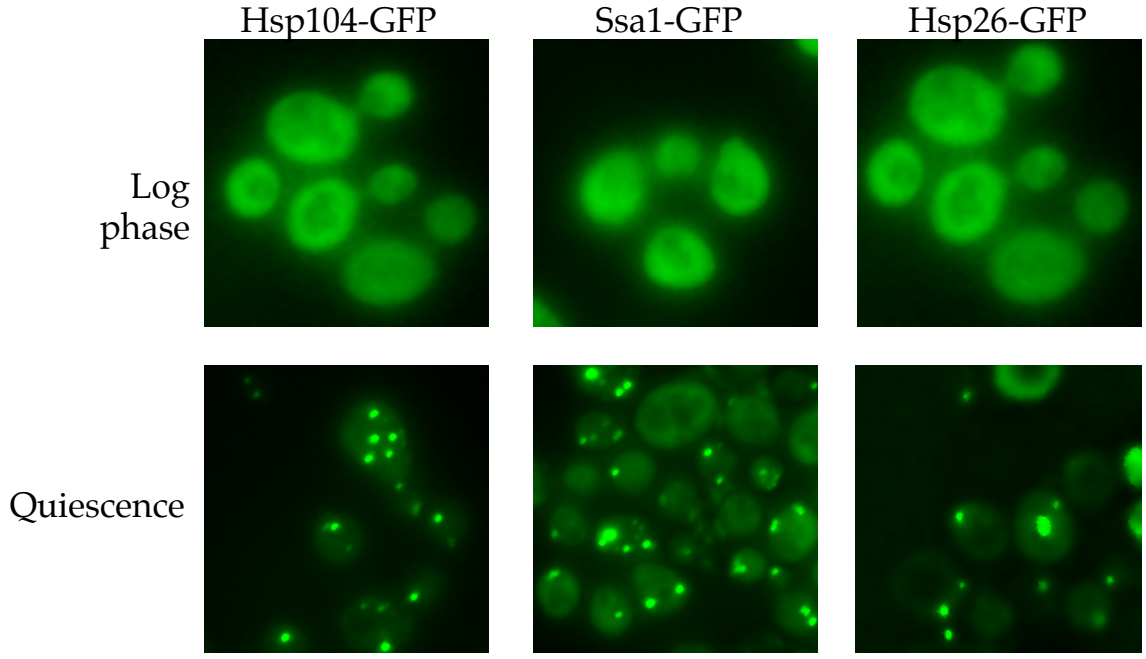
Hsp104, Ssa1 and Hsp26 accumulate in both processing bodies and stress granules

Hsp104 provides cells with thermotolerance and functions together with the Hsp70 chaperone system as well as other small heat shock proteins (sHsps), such as Hsp26, in order to properly refold denatured proteins [51, 52]. To better understand the localization dynamics of Hsp104, I examined the localization of Hsp104 by using a strain with GFP inserted at the *HSP104* genomic locus (39). We first asked whether the subcellular distribution of Hsp104 was affected by stress conditions such as starvation. As shown in Figure 5-1, during the logarithmic phase of growth in rich medium at 30°C, Hsp104-GFP was distributed uniformly throughout the cell. I also examined the localization of two chaperones that function alongside Hsp104; Ssa1, the primary cytosolic yeast Hsp70, and Hsp26, a sHsp that facilitates the disaggregation of aggregated proteins by Hsp104. In a similar manner, both of these chaperones were distributed uniformly throughout the cytoplasm of logarithmic phase cells (Figure 5-1). However, after two days on continued incubation and growth, a distinct concentration was observed for all three chaperones, into discrete foci spread throughout the entire cell (Figure 5-1). Such aggregates have been reported before for Hsp104, and were considered protein aggregates that accumulated in both the nucleus and the cytoplasm of heat-shocked yeast. Hsp104 and Hsp70 have both been implicated in mRNA processing roles such as being required to reactivate mRNA splicing after heat inactivation (105).

Figure 5-1. Hsp104, Ssa1 and Hsp26 accumulate into discrete foci during quiescence

Hps104-GFP, Ssa1-GFP an Hsp26-GFP (genomic fusions) were examined during logarithmic phase and after 3 days of growth, when cells had entered quiescence. Photomicrographs are representative images of GFP localization.

Figure 5-1. Hsp104, Ssa1 and Hsp26 accumulate into discrete foci during quiescence



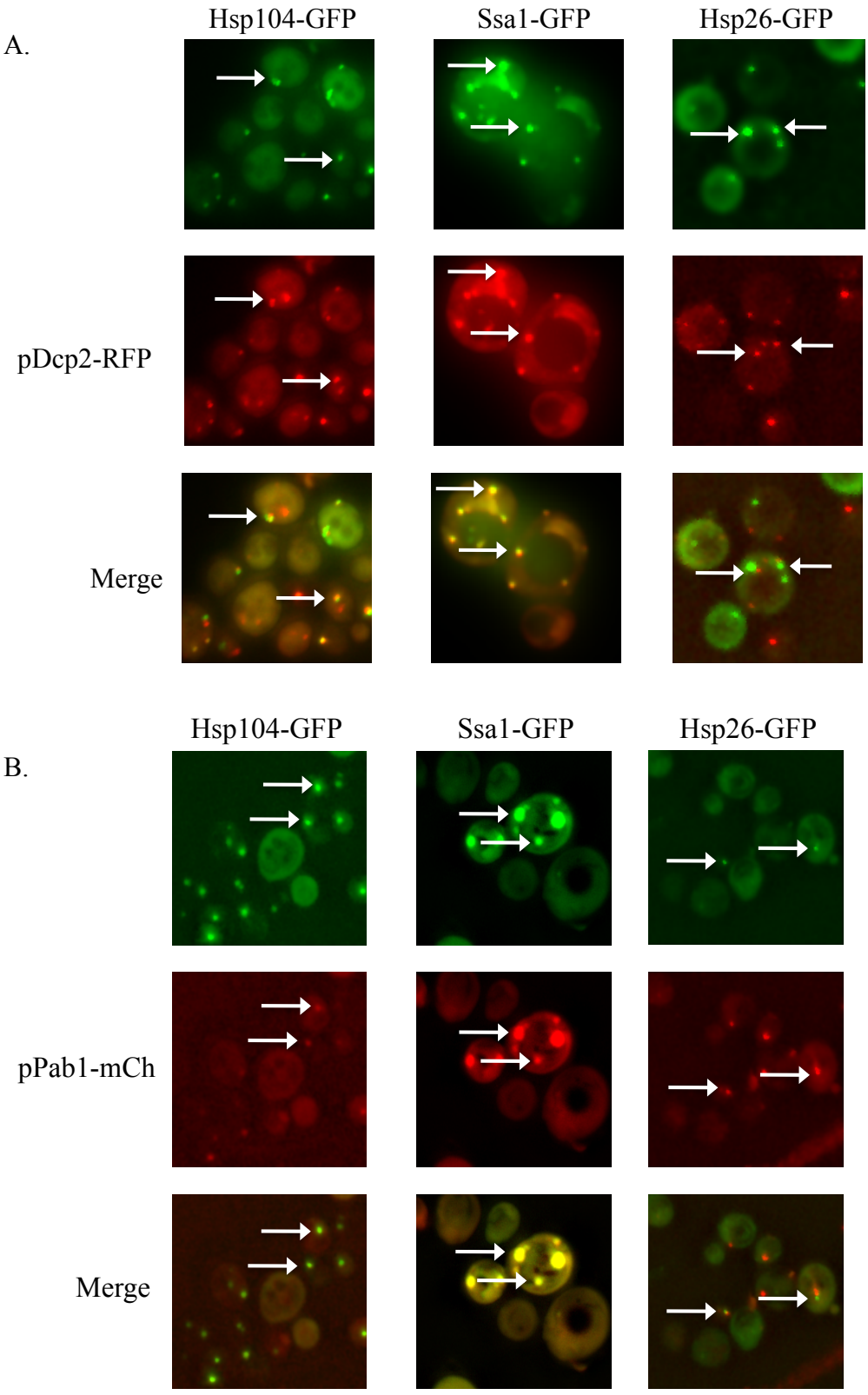
These previous findings and my observation of localization into discrete foci during starvation suggest a possible localization of Hsp104, Ssa1 and Hsp26 into mRNA processing bodies (P-bodies). P-bodies are RNA-protein granules found in eukaryotic cells that contain non-translating mRNAs and proteins involved in mRNA degradation and translational repression (107). P-bodies can be readily observed by stressing yeast cells with distinct stresses such as glucose deprivation or osmotic stress (1M KCl) (111). To examine localization into P-bodies, I examined cells expressing GFP inserted into the genomic loci encoding the three chaperones and transformed with a P-body marker, Dcp2 tagged with the dsRed variant mRFP on a plasmid following glucose deprivation stress. Dcp2, the catalytic subunit of the Dcp1/Dcp2 decapping enzyme complex is a bonafide P-body marker used to localize proteins into P-bodies (106). I observed that all three chaperones co-localized with the decapping enzyme Dcp2, or localize to positions adjacent to P-bodies (Figure 5-2A). This raised the possibility that in addition to localizing into P-bodies, Hsp104, Ssa1 and Hsp26 could also localize into stress granules. Stress granules are mRNPs that form in the cytoplasm of eukaryotic cells when translation is impaired due to stress, in a manner very similar to P-bodies (108). Stress granules and P-bodies are distinguishable by the different protein components found within each granule. While P-bodies serve as mostly as sites for mRNA degradation, stress granules serve as mRNA triage sites. Exchange between the two granules has been observed with P bodies being sites for decisions on mRNA fate, either degrading mRNAs or transferring mRNAs to stress granules for reentry into translation. To examine the localization of the three chaperones into stress granules, I examined the co-localization of Hsp104-GFP, Ssa1-GFP and Hsp26-GFP with a plasmid-borne stress granule marker

Figure 5-2. Hsp104, Ssa1 and Hsp26 are components of both P-bodies and stress granules

A. Strains harboring Hsp104-GFP, Ssa1-GFP and Hsp26-GFP genomic integrations were transformed with a plasmid containing Dcp2-RFP and assayed for GFP co-localization upon glucose deprivation. White arrows point to co-localization.

B. Strains harboring Hsp104-GFP, Ssa1-GFP and Hsp26-GFP genomic integrations were transformed with a plasmid containing Pab1-mCherry and assayed for GFP co-localization upon glucose deprivation. White arrows point to co-localization.

Figure 5-2. Hsp104, Ssa1 and Hsp26 are components of both P-bodies and stress granules



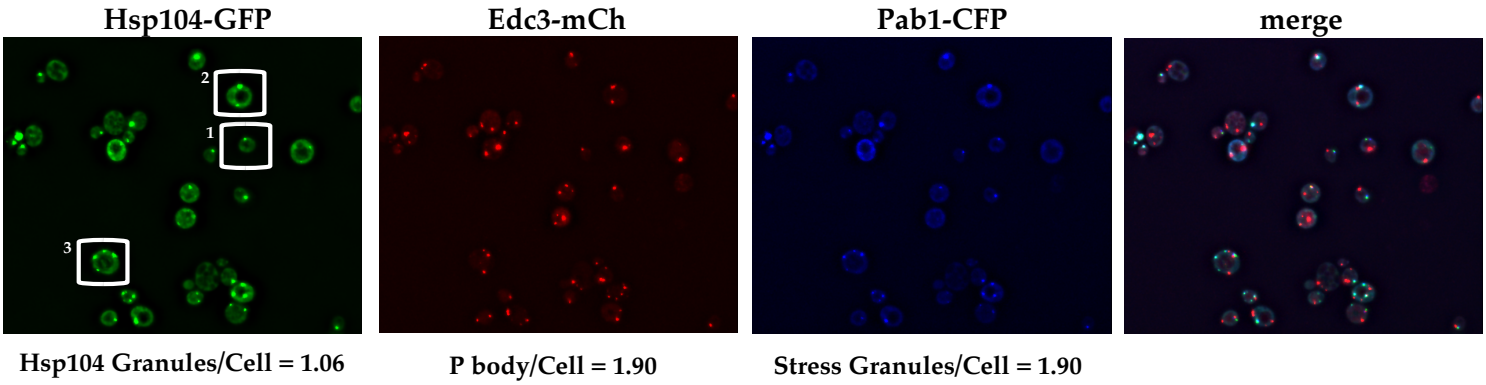
Pab1, tagged with the dsRed variant mCherry, after glucose deprivation stress. Pab1, poly(A) binding protein, mediates interactions between the 5' cap structure and the 3' mRNA poly(A) tail and interacts with translation factor eIF-4G is a commonly used stress granule marker (110). Once again I was able to localize the three chaperones directly into stress granules or adjacent to them, similar to that observed with P-bodies (Figure 5-2B). I interpret these observations to suggest that Hsp104, Ssa1 and Hsp26 might be components of both P-bodies and stress granules.

To differentiate the localization patterns of the three chaperones I utilized a triple fluorescent protein system where Edc3-mCherry (P-body marker) and Pab1-CFP (stress granule marker) were co-expressed from a plasmid in wild-type strains containing chromosomally integrated Hsp104-GFP, Ssa1-GFP or Hsp26-GFP. Following glucose deprivation stress, I observed that all three chaperones concentrated into cytoplasmic foci. When dissecting the localization patterns of different chaperones, I noted four distinct patterns of localization for the three chaperones: 1) the chaperone localizes alone, 2) chaperone localizes into P-bodies, 3) chaperone localizes into stress granules and 4) chaperone localizes with both P-bodies and stress granules, as described in Figure 5-3. The majority of Hsp104 foci localize with Pab1 (40% of total), demonstrating localization primarily into stress granules (Figure 5-3, purple arrows). Hsp104 also co-localizes with Edc3 into P-bodies but this localization pattern is the lowest observed (7% of total, Figure 5-3, yellow arrows). Interestingly, a large subset of the Hsp104 (21% of total) foci localized with both Edc3 and Pab1, which is consistent with previous observations that stress granules and P-bodies in yeast can overlap, suggesting a possible

Figure 5-3. Hsp104 localization patterns

(A) A strain harboring an Hsp104-GFP genomic fusion was transformed with a plasmid containing Edc3-mCherry and Pab1-CFP and assayed for GFP co-localization with mCherry and/or CFP upon glucose deprivation. (B) A closer view of three sample cells, labeled accordingly in A, is shown. Hsp104-GFP co-localized with Edc3-mCherry (yellow arrows), Pab1-CFP (purple arrows), and both Edc3-mCherry and Pab1-CFP (white arrows). Hsp104-GFP was also found in independent foci (green arrows). Edc3-mCherry independent foci can be observed (red arrows) as well as Pab1-CFP independent foci (blue arrows). Analysis of three different individual experiments was done to quantitate the triple fluorescent datasets and assess granule colocalization.

Figure 5-3. Hsp104 localization patterns



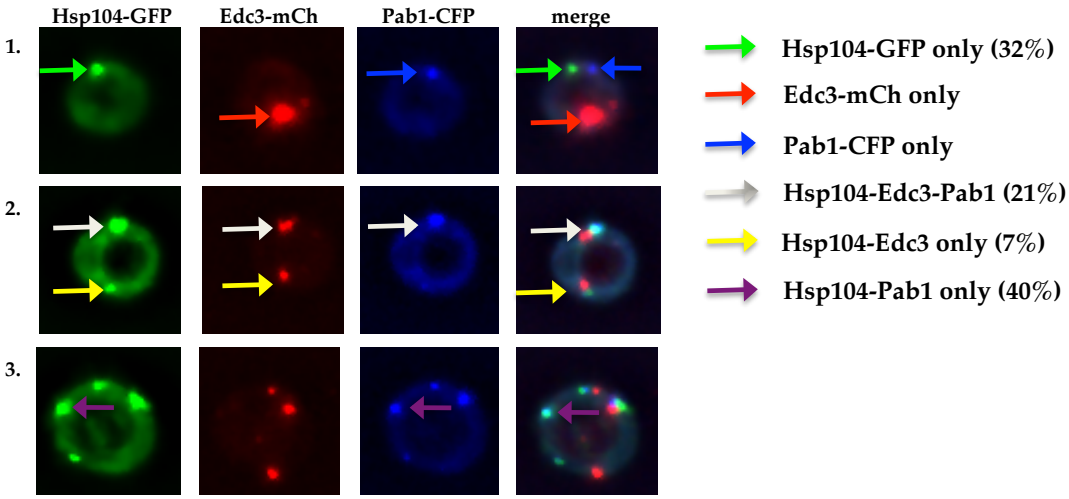
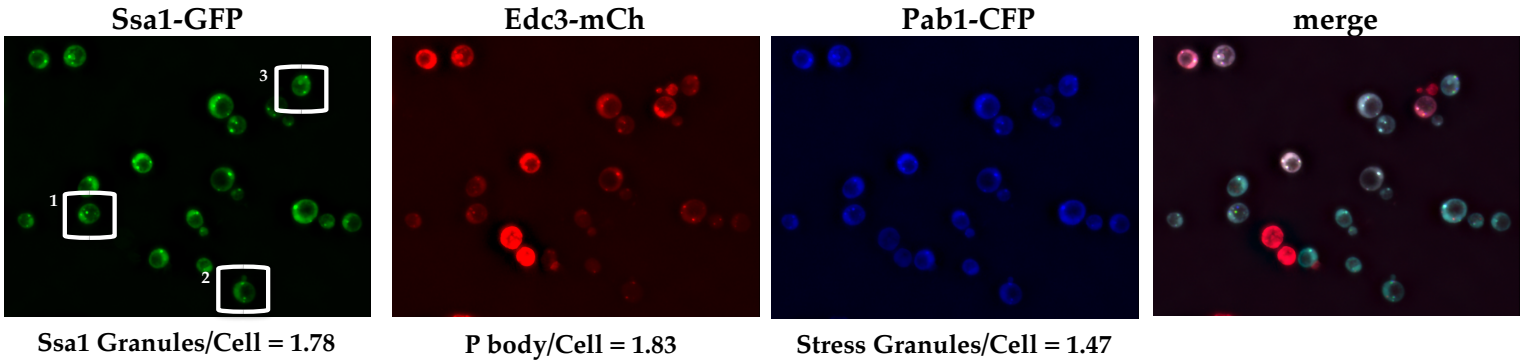


Figure 5-4. Ssa1 localization patterns

(A) A strain harboring an Ssa1-GFP genomic fusion was transformed with a plasmid containing Edc3-mCherry and Pab1-CFP and assayed for GFP co-localization with mCherry and/or CFP upon glucose deprivation. (B) A closer view of three sample cells, labeled accordingly in A, is shown. Ssa1-GFP co-localized with Edc3-mCherry (yellow arrows), Pab1-CFP (purple arrows), and both Edc3-mCherry and Pab1-CFP (white arrows). Ssa1-GFP was also found in independent foci (green arrows). Edc3-mCherry independent foci can be observed (red arrows) as well as Pab1-CFP independent foci (blue arrows). Analysis of three different individual experiments was done to quantitate the triple fluorescent datasets and assess granule colocalization.

Figure 5-4. Ssa1 localization patterns



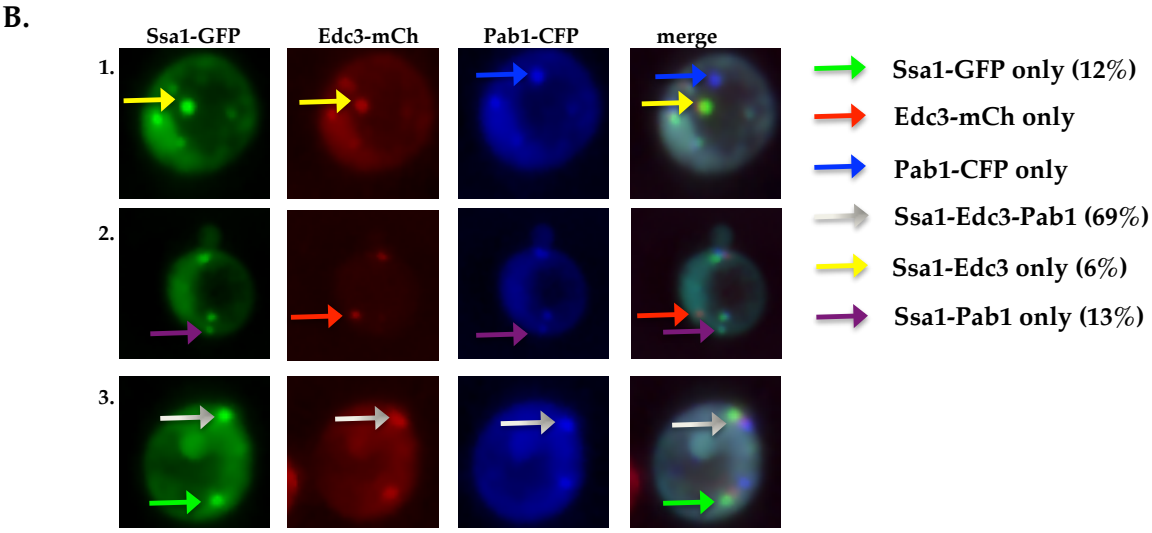
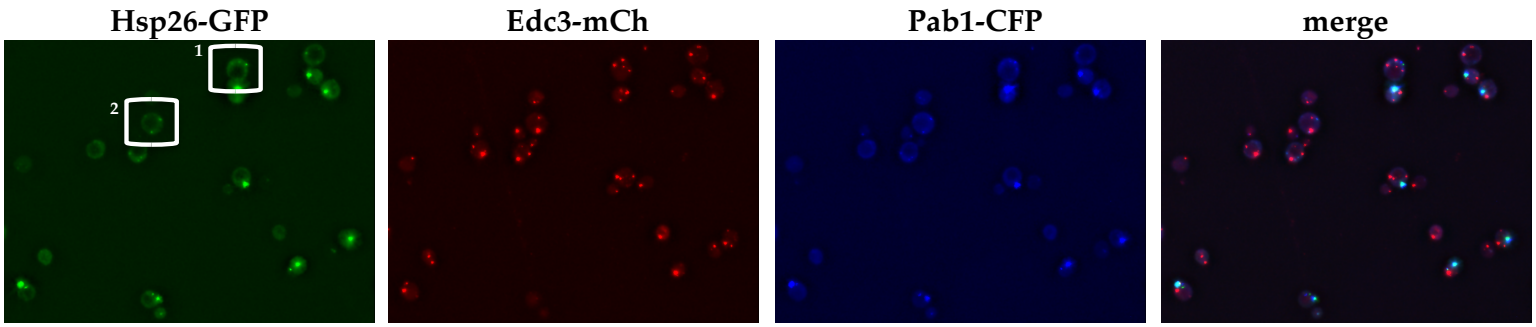


Figure 5-5. Hsp26 localization patterns

(A) A strain harboring an Hsp26-GFP genomic fusion was transformed with a plasmid containing Edc3-mCherry and Pab1-CFP and assayed for GFP co-localization with mCherry and/or CFP upon glucose deprivation. (B) A closer view of two sample cells, labeled accordingly in A, is shown. Ssa1-GFP co-localized with Pab1-CFP (purple arrows), and both Edc3-mCherry and Pab1-CFP (white arrows). Hsp26-GFP was also found in independent foci (green arrows). Edc3-mCherry independent foci can be observed (red arrows) as well as Pab1-CFP independent foci (blue arrows). Analysis of three different individual experiments was done to quantitate the triple fluorescent datasets and assess granule colocalization.

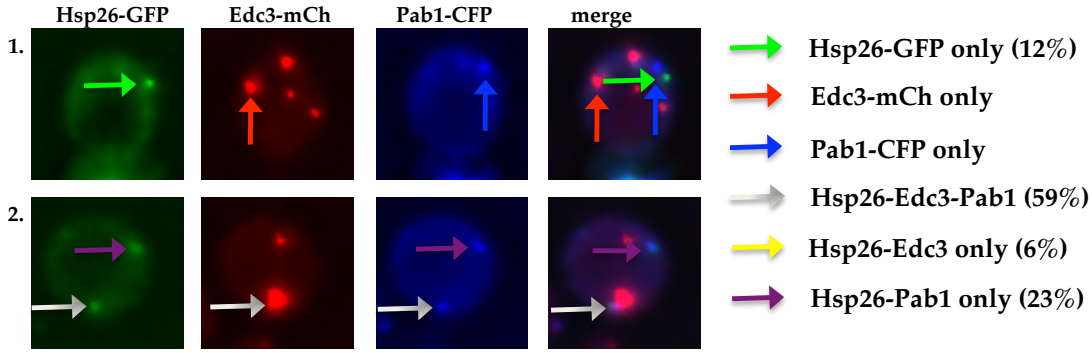
Figure 5-5. Hsp26 localization patterns

A.



Hsp26 Granules/Cell = 1.41 P body/Cell = 1.69 Stress Granules/Cell = 1.20

B.



role for Hsp104 within both RNA granules (Figure 5-3, white arrows). Finally, Hsp104 was observed to be in foci by itself, missing either Edc3 or Pab1. These foci are likely protein aggregates previously reported after heat shock stress (32% of total, Figure 5-3, green arrows). Although we are not stressing cells with heat, localization of Hsp104 into aggregates can be a common response by this specialized chaperone.

Ssa1 demonstrated localization patterns similar to that of Hsp104, localizing into four distinct bodies. Ssa1 co-localized with Edc3 into P-bodies (6% of total, Figure 5-4, yellow arrows), with Pab1 into stress granules (13% of total, Figure 5-4, purple arrows), by itself (12% of total, Figure 5-4, green arrows), and into bodies containing both markers the majority of the time (69% of total, Figure 5-4, white arrows), once again highlighting a possible docking mechanism of both granules in which chaperone activity might be required.

Finally, Hsp26 was also distributed into the four described patterns. Hsp26 co-localizes primarily with Pab1 into stress granules (23% of total, Figure 5-5, purple arrows). Localization was also observed into P-bodies (6% of total, not pictured), found by itself (12% of total, Figure 5-5, green arrows), or found in the P-body - stress granule docked foci (59% of total, Figure 5-5, white arrows). Figure 5-6 depicts the different localization patterns of the three chaperones.

Hsp104, Ssa1 and Hsp26 are required for stress granule formation

Given that Hsp104, Ssa1 and Hsp26 were found to differentially localize to P-bodies, stress granules or a docked combination of both, I wanted to determine whether these chaperones are required for P body and/or stress granule formation. To examine

Figure 5-6. Hsp104, Ssa1, Hsp26 localization patterns

Cartoon depicting the four different localization patterns from Figure 5-3,4,5. Chaperones localize into four patterns: 1) the chaperone localizes alone, 2) chaperone localizes into P-bodies, 3) chaperone localizes into stress granules and 4) chaperone localizes with both P-bodies and stress granules

Figure 5-6. Hsp104, Ssa1, Hsp26 localization patterns

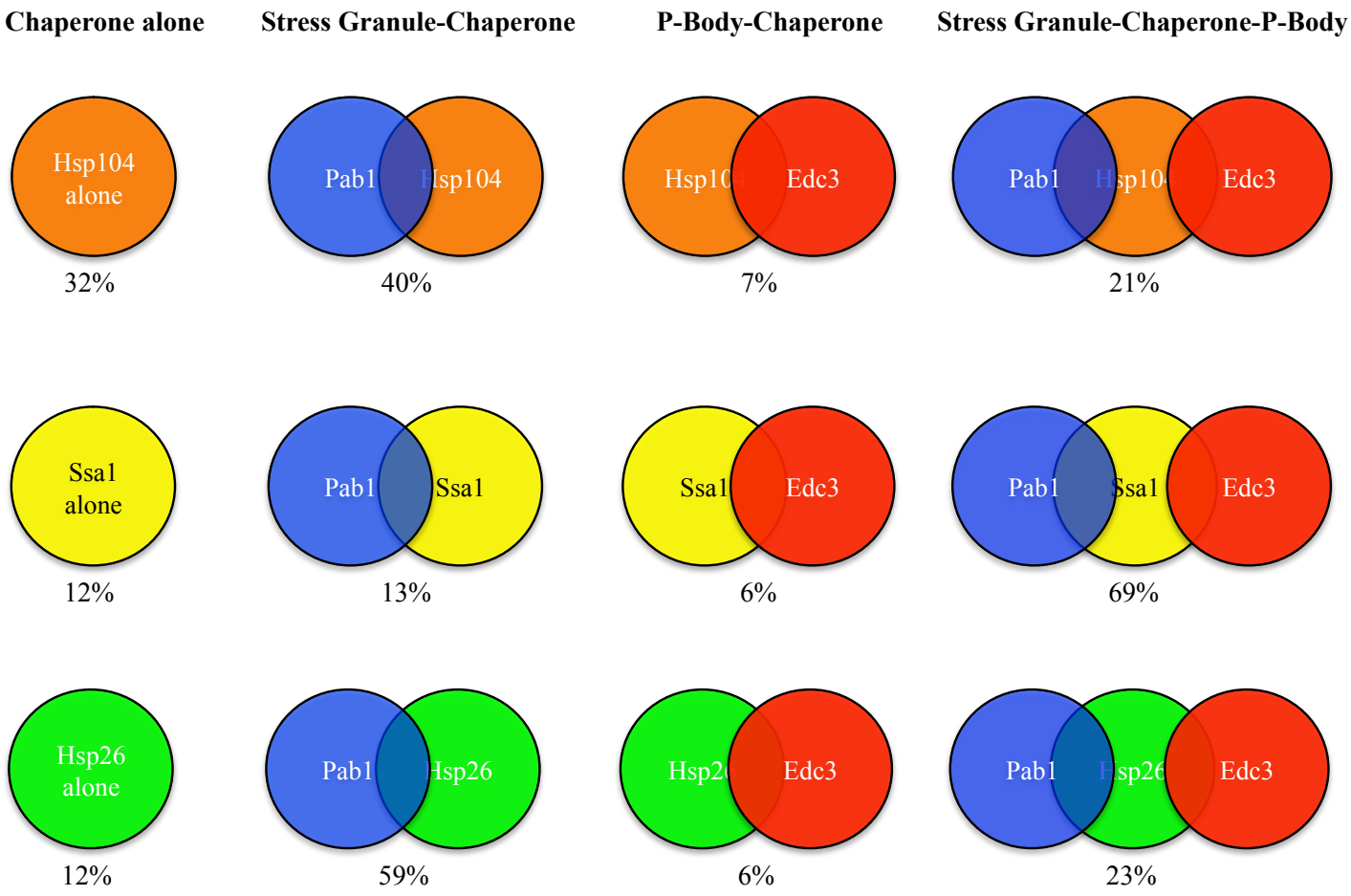
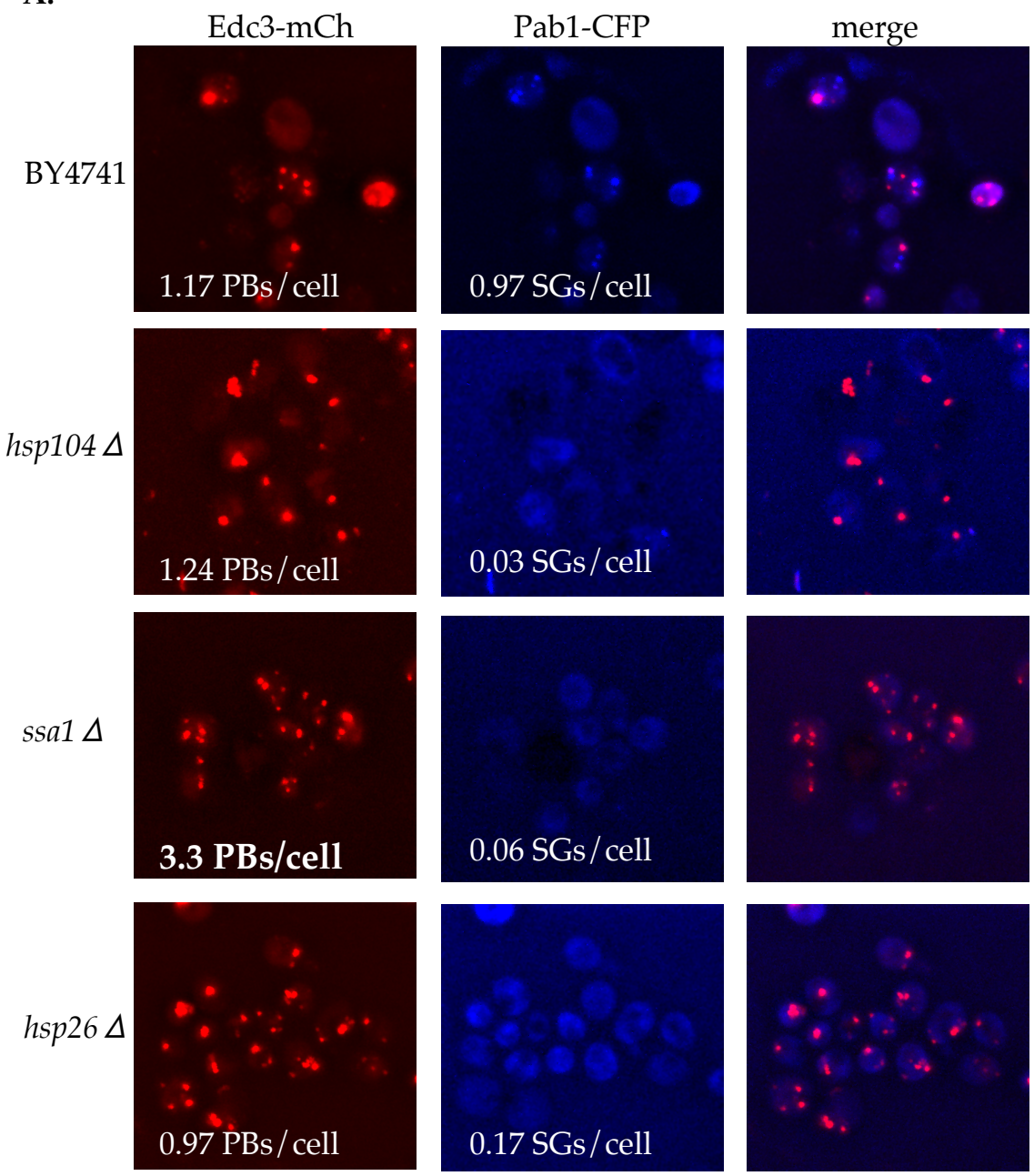


Figure 5-7. Hsp104, Ssa1 and Hsp26 are required for stress granule formation

A. BY4741, *hsp104Δ, ssa1Δ* and *hsp26Δ,* were transformed with a plasmid expressing Edc3-mCherry (P-body marker) and Pab1-CFP (stress granule marker) and examined after glucose deprivation stress. Analysis of three different individual experiments was done to quantitate the fluorescent datasets.

B. BY4741, *hsp104Δ, ssa1Δ* and *hsp26Δ,* were grown to mid-log phase followed by ten minutes of glucose deprivation stress followed by protein extraction analyzed by immunoblot analysis. α-GFP was used to detect Pab1-CFP. Levels of glucose phosphate dehydrogenase were assessed as a loading control (α-GPD).

Figure 5-7. Hsp104, Ssa1 and Hsp26 are required for stress granule formation



this I transformed *hsp104Δ, ssa1Δ* and *hsp26Δ* strains with the dual fluorescent protein system where Edc3-mCherry (P-body marker) and Pab1-CFP (stress granule marker) are co-expressed from the same plasmid and examined the formation of P-bodies and stress granules during glucose deprivation. I observed that all three chaperone deletions destabilized the formation of stress granules (Figure 5-7A). A wild type strain demonstrated an average of 0.97 stress granules per cell in response to ten minutes of glucose deprivation. This percentage was dropped dramatically in the *hsp104Δ, ssa1Δ* and *hsp26Δ* strains to 0.03%, 0.06% and 0.17%, respectively (Figure 5-7A, middle lane). The percentage of P-bodies remained relatively close to wild type in the *hsp104Δ* and *hsp26Δ* strains (BY4741: 1.17 PB/cell, *hsp104Δ*: 1.24 PB/cell, *hsp26Δ*: 0.97 PB/cell). Interestingly, *ssa1Δ* cells exhibited a marked increase in the number of P-bodies compared to wild type, displaying an average of 3.3 P-bodies per cell (Figure 5-7A). Two possible explanations are possible from such a result, either Pab1 was destabilized and degraded or was stable and just delocalized from stress granules. Western blot analysis verified that Pab1-CFP was stable and indeed it is a lack of stress granule formation that is observed in the three chaperone deletions (Figure 5-7B).

Hsp104 is required for the recruitment of Ssa1 and Hsp26 into P-bodies

Since the deletion of any of the three individual chaperones investigated leads to the loss of stress granule formation, I next examined whether there was any loss of chaperone localization to P-bodies in the absence of the different chaperone components. Deletion of *SSA1* did not lead to any observable phenotypes in respect to the recruitment of either Hsp104 nor Hsp26 into P-bodies. Loss of Hsp26 also failed to display any

observable phenotypes in respect to the recruitment of either Hsp104 or Ssa1 into P-bodies. Interestingly, when *HSP104* was not present, both Ssa1 and Hsp26 failed to accumulate into P-bodies (Figure 5-7A,B). Ssa1 altogether failed to accumulate into any observable foci, while foci were visible for Hsp26, but did not co-localize with Dcp2, the P-body marker.

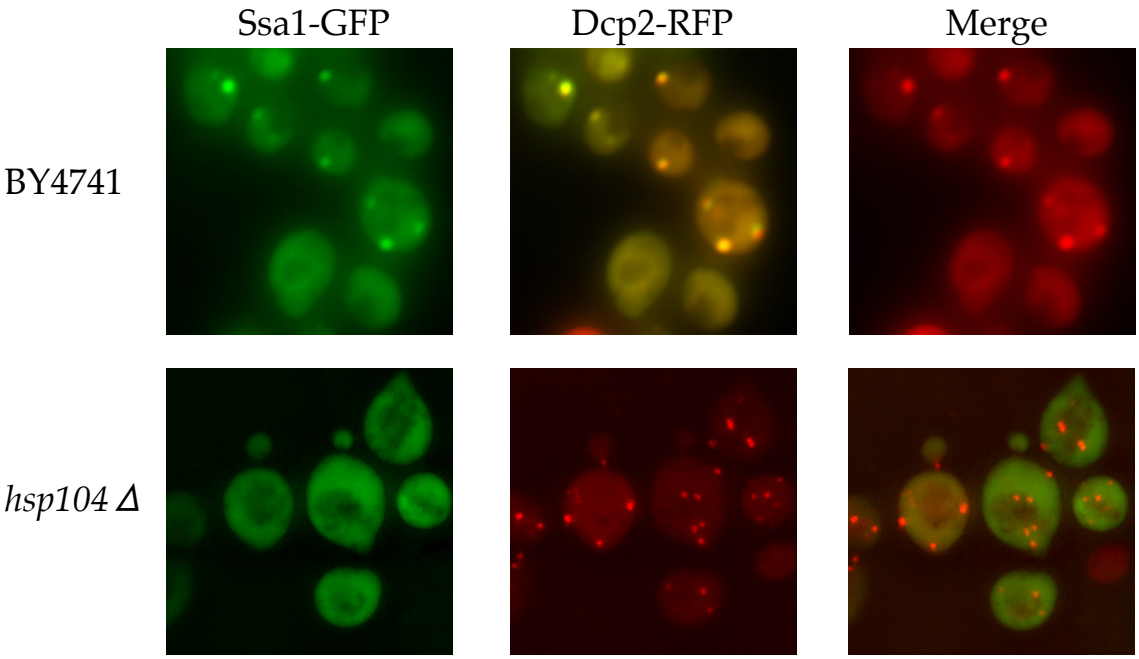
Figure 5-8. Hsp104 is required for the recruitment of Ssa1 and Hsp26 into P-bodies

A. BY4741 and *hsp104Δ* were transformed with pSsa1-GFP and pDcp2-RFP. Cells were grown to mid-log phase and examined after glucose deprivation stress.

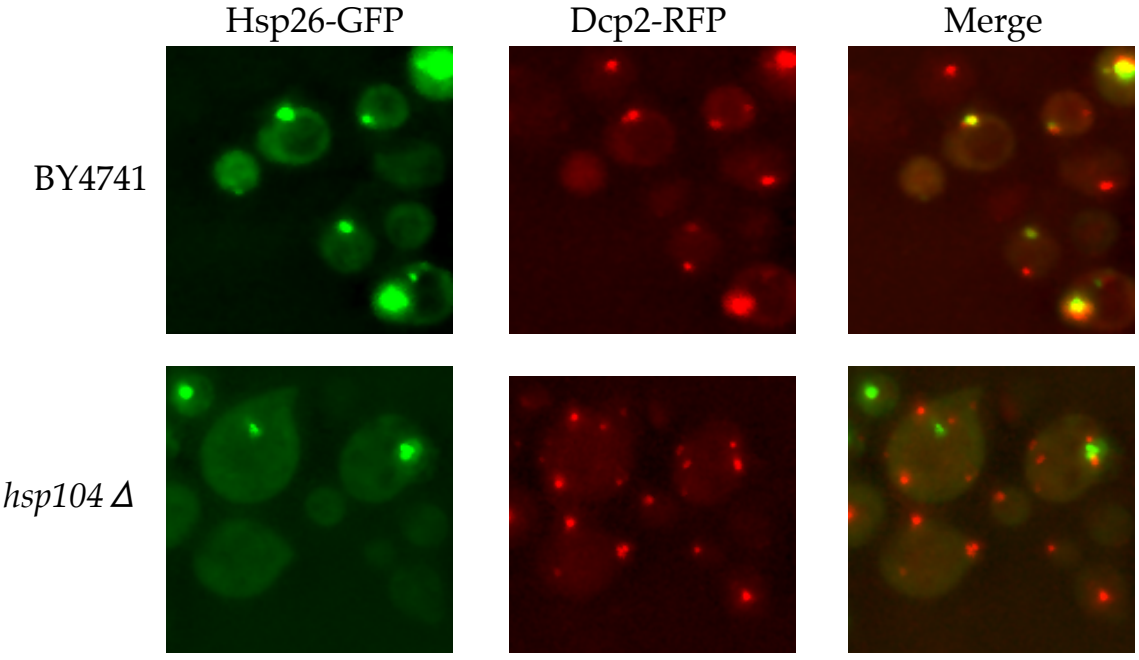
B. BY4741 and *hsp104Δ* were transformed with pHsp26-GFP and pDcp2-RFP. Cells were grown to mid-log phase and examined after glucose deprivation stress. Photomicrographs are representative images.

Figure 5-8. Hsp104 is required for the recruitment of Ssa1 and Hsp26 into P-bodies

A.



B.



Discussion

In this chapter, I describe the localization of three distinct chaperones; Hsp104, Hsp70 and Hsp26, into discrete foci representing P-bodies, stress granules or a combination of the two. In the past few years P-bodies and stress granules, have garnered much attention due to their roles in regulating protein synthesis (107, 108). While both are involved in mRNA processing, have similar sizes and number when examined by fluorescent microscopy, and appear to be conserved from yeast to humans, both have distinct functions. Both granules store mRNAs, yet different fates await the mRNAs, with P-bodies being sites for mRNA degradation and stress granules being sites of mRNA storage and reinitiating translation once conditions become favorable (110). Chaperones are intimately involved in protein homeostasis, yet to date they have not been shown to be involved in either the formation or dispersal of the P-bodies or stress granules observed in the cytoplasm of cells after stress. I observed that Hsp104, Ssa1 (Hsp70) and Hsp26 all demonstrate diffused localization throughout the cell during normal logarithmic growth, yet once cells are deprived of glucose, such as in quiescence, all three chaperones accumulate intro discrete foci within the cytoplasm (Figure 5-1). I demonstrate that Hsp104, Ssa1 and Hsp26 are all components of both P-bodies and stress granules in the yeast, *Saccharomyces cerevisiae* (Figure 5-2). While Hsp104 is not conserved in metazoans, both Hsp70 and Hsp26 have similar orthologs. It will be interesting to investigate whether these two conserved chaperones accumulate into either P-bodies or stress granules in higher eukaryotes.

A closer look at the localization patterns of all three chaperones revealed interesting arrangements of localization into distinct foci. The chaperones accumulated into four

classes of foci: 1) the chaperone localized alone, 2) chaperone localized into P-bodies, 3) chaperone localized into stress granules and 4) chaperone co-localized with both P-body and stress granules. While the chaperones all displayed different localization patterns (Figures 5-3,5-4,5-5), some trends emerged. For example, the chaperones were more often found in the presence of Pab1 in stress granules, or with Pab1 and Edc3 in an RNA granules exhibiting both P-bodies and stress granules co-localized (Figure 5-8). Localization into both bodies simultaneously is exciting since the assembly, disassembly, and transition between the two compartments might prove to be important control points in mRNA metabolism.

Hsp104 can possibly be using its dissaggregase acitivity mechanisms to modulate the formation and dynamics of stress granules. Being mostly associated with stress granules, Hsp104, with the assistance of Hsp70 and Hsp26, might recognize specific proteins in P-bodies that require the disaggregation activity of Hsp104. Once Hsp104 recognizes the aggregated substrates, it will transfer different mRNPs to stress granules for storing or reinitiation of translation once conditions become favorable. This possible mechanism is further supported by the observation that stress granules fail to form in the absence of any one of the three chaperones (Figure 5-7A). Interestingly, deletion of a single Hsp70, Ssa1, in the cell lead to a substantial increase in focal localization of P-bodies. This increase might be due to a lack of Hsp104 stimulation by Ssa1, leading to a lack of disaggregation activity resulting in the increased number of P-bodies. It is interesting to note that *SSA2-4* were all present in the cell, yet deletion of *SSA1* alone demonstrated such a strong phenotype, highlighting the importance of Ssa1 in mRNP granule stability.

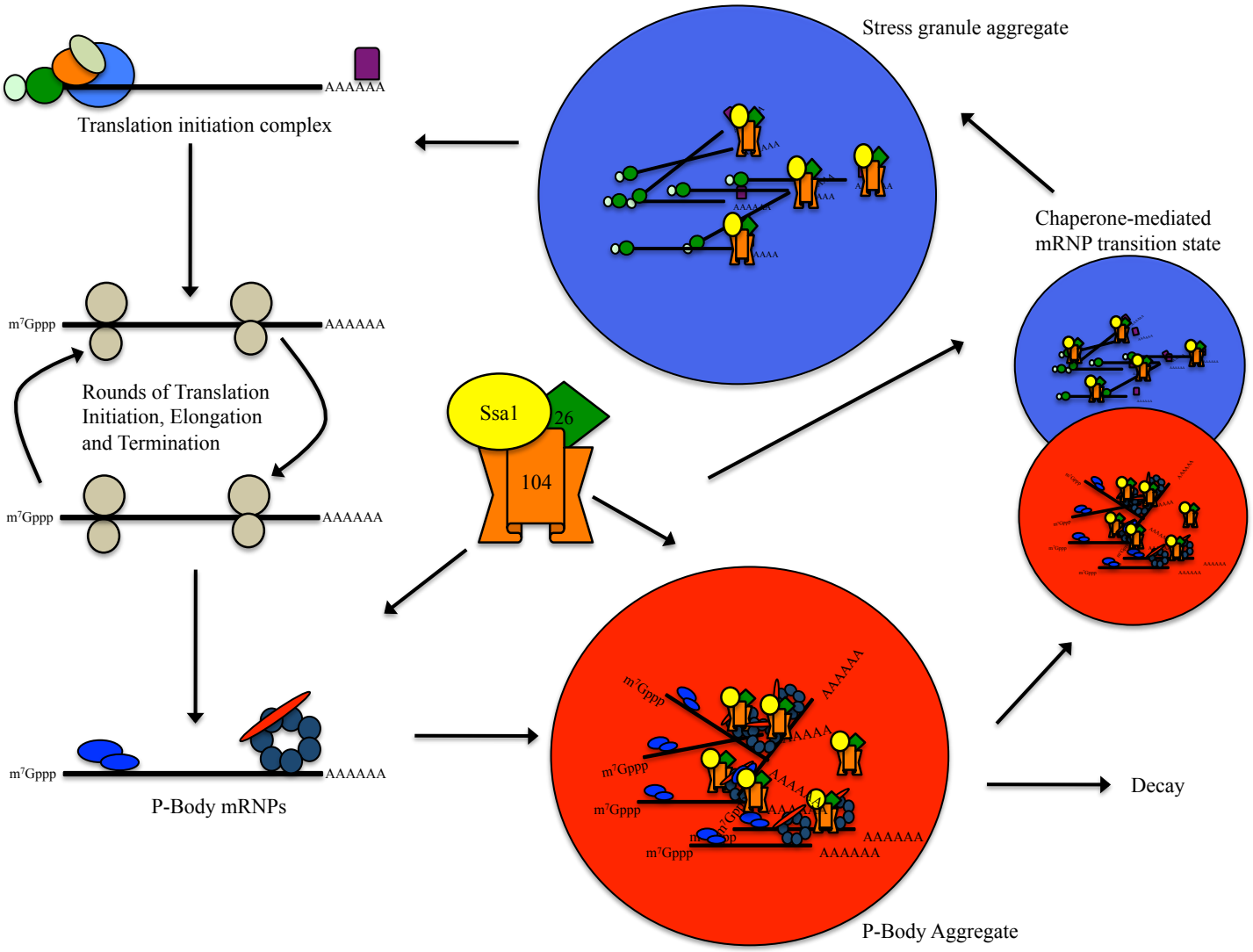
While Hsp104 is not conserved in higher eukaryotes, in yeast it appears responsible for the recruitment of both Ssa1 and Hsp26 into P-bodies upon glucose depravation stress (Figure 5-7). Recruitment of Hsp104 itself might be mediated by P-body components containing Q/N-rich domains leading to aggregation-prone proteins accumulating as P-bodies. It was recently reported that Lsm4, required for efficient decapping and degradation of mRNAs, contains an asparigine rich C-terminus domain that promotes aggregation (112, 113). This aggregation promotes efficient accumulation of Lsm4 and the rest of the Lsm complex (Lsm1-7) into P-bodies. Other P-body components such as Ccr4, Pop2 and Dhh1 all contain Q/N-rich domains that when deleted reduce the number of P bodies in the cell (112, 113). High Q/N content is sufficient to cause protein aggregation, and a subset of Q/N-rich aggregating proteins are capable of stably propagating neurodegenerative diseases as well as prions in yeast (46). Hsp104 and Hsp70 are able to recognize Q/N-rich domains and regulate the formation, growth and elimination of aggregates with these aggregation prone domains found in prions (46). It is then possible that Hsp104 might recognize Q/N-rich domains found on P-body components and this might lead to the localization of Hsp104 into first P-bodies and later stress granules (Figure 5-9).

The data presented in this chapter are significant as they describe a novel localization of three distinct chaperones, Hsp104, Hsp70 and Hsp26 into both P-bodies and stress granules. Localization of chaperones into these small discrete foci highlights a novel role for chaperones in mRNA metabolism. It will be interesting to examine the state of mRNA in the different chaperone deletions that demonstrated a decrease in the formation of stress granules during glucose deprivation. Hsp104 has previously been

Figure 5-9. Model for chaperone involvement in P-body and stress granule assembly.

After exit from translating polysomes, mRNAs are bound by P-body components, forming mRNPs bound to P-bodies. Hsp104, with the assistance of Ssa1 and the sHSP, Hsp26, recongnize P-body components either in the mRNP state or in the P-body aggregate state. Hsp104s disaggregase activity assists in the transfer of mRNPs to stress granules where they will acquire additional translational initiation components such as eIF2 and eIF3, before reinitiating translation. (Color scheme for proteins, P-body: Dcp1/2/Blue, Lsm1-7/Dark BLue, Xrn1/Red. Stress Granules: Pab1/Purple, eIF4G/Light green, eIF4E/Green, 40S/Light blue, eIF2/Beige and eIF3/Orange. Not all proteins present are depicted.)

Figure 5-9. Model for chaperone involvement in P-body and stress granule assembly.



noted to affect mRNA processing events such as being required to reactivate mRNA splicing after heat inactivation (105, 114, 115). The lack of stress granule formation created by the lack of chaperones might lead to a decrease in mRNA stability, especially when Hsp70 is missing from cells, and P-body numbers increase. It will be important to examine the importance of chaperones in the formation of RNA granules in higher eukaryotes. Strong evidence suggests that RNA interference components, not present in budding yeast, interact with P-bodies and imply P-bodies are likely intracellular sites for RNAi (116). Furthermore, it was recently reported that the Hsc70/Hsp90 chaperone machinery might serve as the driving force for the assembly of the RNA-induced silencing complex (117), highlighting a further example of an important role for chaperones in mRNA metabolism by aiding in the assembly or disassembly of mRNP complexes .

Chapter 6: Conclusions and perspectives

Summary and future directions

Quiescent phase nuclear accumulation of Hsp90 – At the onset of these studies, little was known about the subcellar distribution of Hsp90 in the yeast *Saccharomyces cerevisiae*. I reveal for the first time a novel targeting event of Hsp90 into the nucleus upon entrance into quiescence and describe how this targeting event is linked to both chaperone function and spore development.

At the very beginning of these experiments, preliminary data gathered in the lab had demonstrated that Hsc/p82 were localizing to the nucleus of quiescent cells. In order to characterize this nuclear accumulation in more detail, I pursued a number of investigations. First, I decided to analyze the amino acid sequences of Hsc/p82 looking for conserved nuclear export/import sequences. No ubiquitous consensus sequence has been fully described for nuclear translocation beyond the inclusion of hydrophobic or basic stretches of amino acids. I was unable to identify a clear nuclear localization sequence (NLS) or nuclear export sequence (NES) within *HSC82* or *HSP82* after examination of their amino acid sequences. Amino acid analysis actually resulted in approximately four possible regions that could be recognized as nuclear transport sequences. I next decided to pursue the identification of the region within Hsp90 that could be responsible for nuclear transport by using homologous recombination to remove parts of the gene starting from its 5' end. This experimentation again proved difficult as the different mutant alleles that I created were highly unstable demonstrating significant levels of proteolysis. Finally, to generate a non-localizing allele of *HSP82*, a plasmid-borne copy of Hsp82 was subjected to error-prone PCR in order to generate a *HSP82* non-localizing allele. Homologous recombination was used to generate the mutant

plasmids which where then transformed into wild type yeast. Approximately 1,500 independent recombinants were screened for lack of nuclear accumulation during quiescence. Two distinct non-localizing (NL) mutants were isolated and subsequently determined to be able to confer viability when present as the sole source of Hsp90 in the cell demonstrates that nuclear accumulation is not essential for vegetatively growing cells. Detailed analysis of the two NL mutants revealed that substitution of isoleucine 578 to phenylalanine (I578F) in the carboxy terminal domain of the protein was sufficient to block nuclear accumulation of Hsp90 upon glucose exhaustion.

Alongside Hsp90, Sba1, the yeast ortholog to p23, was also found to translocate to the nucleus in response to glucose exhaustion. Sba1/p23 is a late-acting cofactor in the Hsp90 chaperoning cycle, binding to the amino-terminal nucleotide binding domains of both Hsp90 molecules in the functional dimer and stabilizing the chaperone in the ATP-bound state (33, 84). This suggests that a minimal Hsp90 chaperone system is required in the nucleus during periods of limited metabolic activity. In support of this speculation, both Hsp90 and p23 have recently been implicated in exclusively nuclear activities. Freeman and colleagues have shown that Hsp90 and p23 are required to promote telomerase activity by increasing telomerase binding to DNA as well as nucleotide processivity (118, 119). In addition, the Freeman group also demonstrated that loss of Sba1 results in defects in telomere length maintenance, a hallmark of cellular senescence, which differs from quiescence as it an irreversible state (119). To make further relevance for the need of Hsp90 in the nucleus, a report from the laboratory of Mark Ptashne recently showed a delay in transcriptional activation of the galactose transcriptional pathway when components of both the Hsp90 and Hsp70 chaperone

machineries were absent. Once the galactose pathway is activated, inhibitory nucleosomes are rapidly removed from the *GAL1* promoter under wild type conditions, which leads to gene expression. The nucleosome removal is significantly delayed in cells defective in Hsp90 or Hsp70 function (120). It is also know that the Hsp90/Hsp70 chaperone systems are required for proper targeting of client proteins such as p53 and other steroid receptors into the nucleus, facilitating their DNA binding activities (121). These findings highlight important roles for Hsp90 involving both nuclear-localized clients and clients that shuttle from the cytoplasm to the nucleus as part of their functional cycles.

The need for localization of protein chaperones into the nucleus and other subcellular compartments has also been observed with other yeast chaperones. Ursula Stochaj's group demonstrated that under normal growth conditions Ssa4, a yeast Hsp70, shuttles in and out of the nucleus, yet upon various stress conditions it accumulates in the nucleus and redistributes back to the cytoplasm once conditions become more favorable (62). Ssa4 nuclear retention in response to stress is due to relocation of the nuclear export factor, Msn5, which transits to the cytoplasm upon stress. Therefore, nuclear translocation was shown not to be an intrinsic property of Ssa4, but rather an indirect effect of inhibition of the export machinery. On the other hand, Ssb1 is normally a cytoplasmic, ribosome-associated Hsp70 yeast molecular chaperone that functions in the folding of newly made proteins (103). Localization with ribosomes is central to the function of Ssb1, and an NES has been identified which restricts the molecular chaperone to the cytoplasm, maximizing ribosome-binding opportunities possible (122). I have determined that nuclear accumulation of Hsp90 is a gradual response to glucose

exhaustion as cells progress through the diauxic shift into growth by respiration. Interestingly, the disaggregase chaperone Hsp104 interacts with the Hsp90 co-chaperones Cns1, Cpr7 and Sti1 solely in respiring yeast, demonstrating that metabolic conditions may possibly influence chaperone partnering and function (55). Further work will be required to define the nature of the signal and effectors required for nutrient regulation of Hsp90 localization. Preliminary data obtained during the early phase of these studies revealed that the Protein Kinase A (PKA) pathway might be involved in the regulation of Hsp90 nuclear accumulation. Overexpression of Bcy1, a negative regulator of PKA, enhances Hsp90 nuclear accumulation during normal logarithmic growth, in the presence of high concentrations of glucose. Examination of both the TOR and the SNF1 pathways, which regulate entrance into quiescence (123), demonstrated no clear involvement of these pathways leading to the nuclear accumulation of Hsp90.

My examinations revealed that under favorable conditions, Hsp90 likely shuttles between the cytoplasm and nucleus, yet only upon glucose exhaustion does this chaperone accumulate in the nucleus. The yeast *SRP1/KAP95* gene pair encode for the essential orthologs of the established α/β importin system required for the transport of non-diffusible proteins into the nucleus (87). By using a temperature sensitive allele of *KAP95*, I identified this importin as being responsible for the nuclear trafficking of Hsp90 and Sba1.

Reduced Hsp90 nuclear accumulation is coincident with growth and developmental defects. - I also describe a new role for the Hsp90 chaperone in development based on nutrient-responsive nuclear accumulation. If Hsp90 localizes to the mitotic nucleus during glucose starvation, I then reasoned that Hsp90 should localize to the nuclei of the

four haploid spore progeny produced during sporulation. I observed that Hsp90 indeed localizes to all four spore nuclei, consistent with our previous data that Hsp90 nuclear localization is a specific response to nutrient starvation, and not a general stress response. Surprisingly, sporulating cells expressing the *hsp82-I578F-GFP* non-localizing mutant failed to produce mature spores. Instead, the mutant exhibited a variety of sporulation defects ranging from atypically large cells apparently devoid of spores to cells with compartments indicative of immature, disorganized spores. The I578F mutation might lead to a change in the tertiary or quaternary structure of Hsp90, preventing nuclear accumulation. This possibility is supported by our finding that two previously characterized Hsp90 hypomorphic mutant alleles, Hsp90-G313N, and to a lesser extent Hsp90-E431K, likewise failed to localize to the nucleus during quiescence, linking Hsp90 functional status to its subcellular localization. At this time, I cannot completely divorce Hsp90 function from subcellular localization or truly conclude which is the deficiency responsible for the lack of proper development. However, it is interesting to note the convergence of localization and function phenotypes; in a search for mislocalizing *HSP82* mutants I uncovered a functionally impaired allele, and two mutants previously isolated on the basis of loss of function were found by me to mislocalize. A formal test of the role of nuclear accumulation during quiescence will require further in-depth study.

The dramatic defects observed in spore formation and maturation in cells genetically or pharmacologically depleted of Hsp90 activity strongly implicates this protein chaperone in the process of yeast meiotic development, and suggests the existence of one or more formerly unappreciated client proteins involved in this

important biological process. Carefully regulated gene expression during sporulation is required to properly orchestrate yeast development. Remarkably, I did not observe defects in meiotic nuclear division, which suggests that Hsp90 plays a critical role downstream of meiosis and is likely important in proper spore wall synthesis. Spore wall assembly in budding yeast depends on proper signaling by the mitogen activated protein kinase (MAPK), Smk1 (101). Different Smk1 mutant alleles have been described to result in improper spore wall formation in a manner comparable to what I observed in our I578F mutant (101). Future work will be required to precisely determine the identity of the Hsp90 clients responsible for the panoply of defects I have documented during spore assembly when the protein chaperone is prevented from localizing to either the pre-meiotic or spore nuclei. Our results suggest a functional and perhaps spatial role for the Hsp90 chaperone in a developmental process in the brewing yeast *S. cerevisiae.*

It is interesting that, despite the depth of knowledge in the field of chaperone biology, such an important function has remained uncharacterized for so long. Investigation of chaperone function in yeast development might aid in better understanding an important biological process. Insights into better understanding other developmental process in yeast will help better understand the function of Hsp90 in fungal development. Leah Cowen and colleagues have documented that Hsp90 in the fungal pathogen *Candida albicans* controls not only the cellular circuitry required for drug resistance but also plays a key role in the morphogenetic transition from yeast to filamentous growth, a developmental process that is crucial for virulence (124). It will be important to examine whether Hsp90 is required for a similar event that occurs in selected strains of *S. cerevisiae* (Σ1278b) that exhibit pseudohyphal differentiation when

starved for nitrogen (88). In lieu of such an easily scorable phenotype, it will prove beneficial to further attempt to characterize factors that might be involved in both the nuclear accumulation and the developmental aspects of Hsp90. A high-copy suppressor screen for genes that are either able to restore nuclear accumulation or proper development of cells bearing the I578F mutation might prove very insightful.

Hsp104, Ssa1 and Hsp26 are components of processing bodies and stress granules – It has previously been reported that yeast cells accumulate electron-dense aggregates that are likely aggregated proteins in both the cytoplasm and nucleus of the cell (51). These aggregates dissolve during recovery from heat shock but only when a functional Hsp104 is expressed (51). Furthermore, Hsp104 has been shown to accumulate in the periphery of aggregates both in the cytoplasm and nucleus of heat-stressed cells (63). I have demonstrated that Hsp104, Hsp70 and Hsp26 localize into discrete foci representing processing bodies (P-bodies), stress granules or a combination of the two mRNP granules. Both mRNP granules store mRNAs, yet different fates await the mRNAs, with P-bodies being sites for mRNA degradation and stress granules being sites of mRNA triage, reinitiating translation when conditions become favorable. Chaperones are intimately involved in protein homeostasis, yet they have not been shown to be involved in either the formation or dispersal of the different RNA aggregates observed in the cytoplasm of cells after stress. A closer look at the localization patterns of all three chaperones revealed interesting patterns of chaperone and RNA granule localization. The chaperones could be divided into four classes of foci: 1) the chaperone localized alone, 2) chaperone localized into P-bodies, 3) chaperone localized into stress granules and 4)

chaperone localized with both P-body and stress granules. While the chaperones all displayed different localization patterns they were more often found in the presence of Pab1 in stress granules, or with Pab1 and Edc3 in an RNA granules exhibiting both P-bodies and stress granules co-localized (Figure 5-6).

Being predominantly associated with stress granules, Hsp104, with the assistance of Hsp70 and Hsp26, might recognize specific proteins in P-bodies that require the disaggregation activity of Hsp104 in manner reminiscent of the severing of prion fibrils by Hsp104. In vitro, Hsp104 is capable of severing yeast fibers by interacting with an exposed unstructured tag on a prion protomer (46). Prion protomers are extracted from the aggregated amyloid by unfolding and translocation through the channel of Hsp104 and released in an ATP dependent manner. Although the Hsp70 system is not required for this activity, it and other co-chaperones such as small heat shock proteins indeed modulate this activity in vivo (46). The overall process results in twice the number of prions or "seeds" that can grow into new prion fibers. In a similar manner I envision that once Hsp104 recognizes the assembled substrates within P-bodies, it may transfer different mRNPs to stress granules for storage or reinitiation of translation once conditions become favorable, such as addition of nutrients to quiescent cells. This possible mechanism is further supported by the observation that stress granules fail to form in the absence of any one of the three chaperones.

Hsp104 appears responsible for the recruitment of both Ssa1 and Hsp26 into P-bodies upon glucose deprivation stress. It will be interesting to examine whether a functional copy of Hsp104 is required for the recruitment of Ssa1 and Hsp26 to these mRNP granules. We are currently asking this question using two different *HSP104*

alleles. The first mutant is a single amino acid substitution, Y662A (125), that abolishes the ability of Hsp104 to refold substrates while maintaining its ATPase activity and fails to provide thermotolerance to cells. The second mutant, termed 'trap,' or Double Walker B (DWB) mutant is a double amino acid substitution, E285Q/E687Q in the Walker ATP binding motif, which still can bind ATP but cannot hydrolyze it, hence it 'traps' substrates (58). I predict that the 'trap' mutant might increase the number of P-bodies in the cell due to a lack of Hsp104 disaggregase activity, 'traping' Hsp104 in P-bodies and at the same time decrease the number of stress granules. It will also be important to test whether these two distinct mutant alleles are themselves correctly targeted to granules.

Recruitment of Hsp104 itself might be mediated by P-body components containing Q/N-rich domains, or prion-like domains, leading to aggregation-prone proteins accumulating as P-bodies. It was recently reported that Lsm4, required for efficient decapping and degradation of mRNAs, contains an asparigine rich C-terminal domain that promotes Lsm4 multimerization (112, 113). This aggregation promotes efficient accumulation of Lsm4 and the rest of the Lsm complex (Lsm1-7) into P-bodies for proper mRNA degradation. Other P-body components such as Ccr4, Pop2 and Dhh1 all contain Q/N-rich domains that when deleted reduce the number of P bodies in the cell (112, 113). High Q/N content is sufficient to cause protein aggregation, and a subset of Q/N-rich aggregating proteins such as beta-amyloid fibers responsible for Alzheimer's disease are capable of stably propagating neurodegenerative diseases as well as prions in yeast (17, 19, 46). Hsp104 and Hsp70 are able to recognize Q/N-rich domains and regulate the formation, growth and elimination of aggregates with these prion-like domains. It is then possible that Hsp104 might recognize Q/N-rich domains found on P-body components

and this might lead to the localization of Hsp104 into first P-bodies and later stress granules (Figure 5-9). Work in the lab is currently aimed at better understanding the proteins required for the accumulation of Hsp104 and the other chaperones into both P bodies and stress granules.

I describe a novel localization of three distinct chaperones; Hsp104, Hsp70 and Hsp26 into both P-bodies and stress granules. Localization of chaperones into these small discrete foci highlights a possibly overlooked role for chaperones in mRNA metabolism. It will be essential to examine the state of mRNA in the different chaperone deletions that demonstrated a decrease in the formation of stress granules during glucose deprivation. It is interesting to note that Ssa1 regulates the turnover of the MFA2 transcript through its AU-rich 3' untranslated region (126). This stabilization occurs as a result in a decrease in deadenylation suggesting that Hsp70 is required for activation of the turnover pathway (126). Hsp104 has previously been noted to affect mRNA processing roles such as being required to reactivate mRNA splicing after heat inactivation (105). The lack of stress granule formation caused by the lack of chaperones might lead to a decrease in mRNA stability, especially when Hsp70 is missing from cells, and P-body numbers increase. It will be important to examine the requirement of chaperones in the formation of RNA granules in higher eukaryotes. Early studies on molecular chaperone localization in chicken embryo fibrioblasts show that a sHSP, Hsp24, accumulates into phase-dense granules similar to P-bodies and stress granules (127). Strong evidence suggests that RNA interference components, not present in budding yeast, are interacting with P-bodies in metazoan cells and P-bodies are likely focal sites for RNAi (116). Furthermore, it was recently reported that the Hsc70/Hsp90

chaperone machinery might serve as the driving force for the assembly of the RNA-induced silencing complex (117), highlighting a further example of an important role for chaperones in mRNA metabolism by aiding in the assembly or disassembly of mRNP complexes.

Concluding remarks – My thesis work has significantly advanced the field of molecular chaperone biology by characterizing two novel roles for central chaperones in the yeast *S. cerevisiae*. I uncovered a novel targeting event of Hsp90 into the nucleus upon entrance into quiescence and its role in yeast development. My work provides a foundation on which to develop a better understanding of the precise molecular mechanism being employed by Hsp90 to help cells properly develop. I also demonstrate that three related chaperones, Hsp104, Ssa1 and Hsp26 accumulate into mRNP granules and are required for the formation of stress granules and regulation of P-body abundance. The results of these studies can now be applied to experiments to better understand the expanding field of P-body biology and to examine the importance of these factors in the formation of mRNP granules in higher eukaryotes.

Bibliography

1. Hecker M, Volker U. General stress response of Bacillus subtilis and other bacteria. Advances in microbial physiology 2001;44:35-91.

2. Kultz D. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. The Journal of experimental biology 2003;206(Pt 18):3119-3124.

3. Kultz D. Molecular and evolutionary basis of the cellular stress response. Annual review of physiology 2005;67:225-257.

4. Gasch AP, Werner-Washburne M. The genomics of yeast responses to environmental stress and starvation. Functional & integrative genomics 2002;2(4-5):181-192.

5. Trott A, and K.A. Morano The yeast response to heat shock. In Yeast Stress Responses - S Hohmann and PWH mager (ed) 2003;1:71-119.

6. Hohmann S, and W.H. Mager. Introduction Chapter. In Yeast Stress Responses - S Hohmann and PWH mager (ed) 2003;1:1-9.

7. Balch WE, Morimoto RI, Dillin A, Kelly JW. Adapting proteostasis for disease intervention. Science (New York, NY 2008;319(5865):916-919.

8. Dai C, Whitesell L, Rogers AB, Lindquist S. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. Cell 2007;130(6):1005-1018.

9. Westerheide SD, Anckar J, Stevens SM, Jr., Sistonen L, Morimoto RI. Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. Science (New York, NY 2009;323(5917):1063-1066.

10. Harrison CJ, Bohm AA, Nelson HC. Crystal structure of the DNA binding domain of the heat shock transcription factor. Science (New York, NY 1994;263(5144):224-227.

11. Giardina C, Lis JT. Dynamic protein-DNA architecture of a yeast heat shock promoter. Molecular and cellular biology 1995;15(5):2737-2744.

12. Morano KA, Thiele DJ. Heat shock factor function and regulation in response to cellular stress, growth, and differentiation signals. Gene expression 1999;7(4-6):271-282.

13. Picard D. Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci 2002;59(10):1640-1648.

14. Caplan AJ. Hsp90's secrets unfold: new insights from structural and functional studies. Trends in cell biology 1999;9(7):262-268.

15. Pearl LH, Prodromou C. Structure and mechanism of the Hsp90 molecular chaperone machinery. Annual review of biochemistry 2006;75:271-294.

16. Pratt WB. The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY 1998;217(4):420-434.

17. Ansar S, Burlison JA, Hadden MK, Yu XM, Desino KE, Bean J, Neckers L, Audus KL, Michaelis ML, Blagg BS. A non-toxic Hsp90 inhibitor protects neurons from Abeta-induced toxicity. Bioorganic & medicinal chemistry letters 2007;17(7):1984-1990.

18. Dickey CA, Kamal A, Lundgren K, Klosak N, Bailey RM, Dunmore J, Ash P, Shoraka S, Zlatkovic J, Eckman CB, Patterson C, Dickson DW, Nahman NS, Jr., Hutton M, Burrows F*, et al.* The high-affinity HSP90-CHIP complex recognizes and selectively

degrades phosphorylated tau client proteins. The Journal of clinical investigation 2007;117(3):648-658.

19. Evans CG, Wisen S, Gestwicki JE. Heat shock proteins 70 and 90 inhibit early stages of amyloid beta-(1-42) aggregation in vitro. The Journal of biological chemistry 2006;281(44):33182-33191.

20. Luo W, Dou F, Rodina A, Chip S, Kim J, Zhao Q, Moulick K, Aguirre J, Wu N, Greengard P, Chiosis G. Roles of heat-shock protein 90 in maintaining and facilitating the neurodegenerative phenotype in tauopathies. Proceedings of the National Academy of Sciences of the United States of America 2007;104(22):9511-9516.

21. Luo W, Rodina A, Chiosis G. Heat shock protein 90: translation from cancer to Alzheimer's disease treatment? BMC neuroscience 2008;9 Suppl 2:S7.

22. Neckers L, Mimnaugh E, Schulte TW. Hsp90 as an anti-cancer target. Drug Resist Updat 1999;2(3):165-172.

23. Cowen LE, Carpenter AE, Matangkasombut O, Fink GR, Lindquist S. Genetic architecture of Hsp90-dependent drug resistance. Eukaryotic cell 2006;5(12):2184-2188.

24. Rutherford SL, Lindquist S. Hsp90 as a capacitor for morphological evolution. Nature 1998;396(6709):336-342.

25. Sangster TA, Lindquist S, Queitsch C. Under cover: causes, effects and implications of Hsp90-mediated genetic capacitance. Bioessays 2004;26(4):348-362.

26. Sangster TA, Queitsch C. The HSP90 chaperone complex, an emerging force in plant development and phenotypic plasticity. Curr Opin Plant Biol 2005;8(1):86-92.

27. Pearl LH, Prodromou C. Structure and in vivo function of Hsp90. Current opinion in structural biology 2000;10(1):46-51.

28. Borkovich KA, Farrelly FW, Finkelstein DB, Taulien J, Lindquist S. hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol Cell Biol 1989;9(9):3919-3930.

29. Richter K, Muschler P, Hainzl O, Buchner J. Coordinated ATP hydrolysis by the Hsp90 dimer. The Journal of biological chemistry 2001;276(36):33689-33696.

30. Pearl LH, Prodromou C. Structure, function, and mechanism of the Hsp90 molecular chaperone. Advances in protein chemistry 2001;59:157-186.

31. Chang HC, Nathan DF, Lindquist S. In vivo analysis of the Hsp90 cochaperone Sti1 (p60). Molecular and cellular biology 1997;17(1):318-325.

32. Felts SJ, Toft DO. p23, a simple protein with complex activities. Cell Stress Chaperones 2003;8(2):108-113.

33. Fang Y, Fliss AE, Rao J, Caplan AJ. SBA1 encodes a yeast hsp90 cochaperone that is homologous to vertebrate p23 proteins. Molecular and cellular biology 1998;18(7):3727-3734.

34. Arevalo-Rodriguez M, Wu X, Hanes SD, Heitman J. Prolyl isomerases in yeast. Front Biosci 2004;9:2420-2446.

35. Vaughan CK, Mollapour M, Smith JR, Truman A, Hu B, Good VM, Panaretou B, Neckers L, Clarke PA, Workman P, Piper PW, Prodromou C, Pearl LH. Hsp90-dependent activation of protein kinases is regulated by chaperone-targeted dephosphorylation of Cdc37. Molecular cell 2008;31(6):886-895.

36. Felts SJ, Owen BA, Nguyen P, Trepel J, Donner DB, Toft DO. The hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties. The Journal of biological chemistry 2000;275(5):3305-3312.

37. Richter K, Reinstein J, Buchner J. A Grp on the Hsp90 mechanism. Molecular cell 2007;28(2):177-179.

38. Pratt WB, Silverstein AM, Galigniana MD. A model for the cytoplasmic trafficking of signalling proteins involving the hsp90-binding immunophilins and p50cdc37. Cell Signal 1999;11(12):839-851.

39. Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK. Global analysis of protein localization in budding yeast. Nature 2003;425(6959):686-691.

40. Erzberger JP, Mott ML, Berger JM. Structural basis for ATP-dependent DnaA assembly and replication-origin remodeling. Nature structural & molecular biology 2006;13(8):676-683.

41. Parsell DA, Sanchez Y, Stitzel JD, Lindquist S. Hsp104 is a highly conserved protein with two essential nucleotide-binding sites. Nature 1991;353(6341):270-273.

42. Schirmer EC, Lindquist S, Vierling E. An Arabidopsis heat shock protein complements a thermotolerance defect in yeast. The Plant cell 1994;6(12):1899-1909.

43. Zenthon JF, Ness F, Cox B, Tuite MF. The [PSI+] prion of Saccharomyces cerevisiae can be propagated by an Hsp104 orthologue from Candida albicans. Eukaryotic cell 2006;5(2):217-225.

44. Sanchez Y, Lindquist SL. HSP104 required for induced thermotolerance. Science (New York, NY 1990;248(4959):1112-1115.

45. Grably MR, Stanhill A, Tell O, Engelberg D. HSF and Msn2/4p can exclusively or cooperatively activate the yeast HSP104 gene. Molecular microbiology 2002;44(1):21-35.

46. Doyle SM, Wickner S. Hsp104 and ClpB: protein disaggregating machines. Trends in biochemical sciences 2009;34(1):40-48.

47. Sanchez Y, Taulien J, Borkovich KA, Lindquist S. Hsp104 is required for tolerance to many forms of stress. The EMBO journal 1992;11(6):2357-2364.

48. Glover JR, Lindquist S. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell 1998;94(1):73-82.

49. Lindquist S, DebBurman SK, Glover JR, Kowal AS, Liu JJ, Schirmer EC, Serio TR. Amyloid fibres of Sup35 support a prion-like mechanism of inheritance in yeast. Biochemical Society transactions 1998;26(3):486-490.

50. Sanchez Y, Parsell DA, Taulien J, Vogel JL, Craig EA, Lindquist S. Genetic evidence for a functional relationship between Hsp104 and Hsp70. Journal of bacteriology 1993;175(20):6484-6491.

51. Parsell DA, Kowal AS, Singer MA, Lindquist S. Protein disaggregation mediated by heat-shock protein Hsp104. Nature 1994;372(6505):475-478.

52. Cashikar AG, Duennwald M, Lindquist SL. A chaperone pathway in protein disaggregation. Hsp26 alters the nature of protein aggregates to facilitate reactivation by Hsp104. The Journal of biological chemistry 2005;280(25):23869-23875.

53. Amoros M, Estruch F. Hsf1p and Msn2/4p cooperate in the expression of Saccharomyces cerevisiae genes HSP26 and HSP104 in a gene- and stress type-dependent manner. Molecular microbiology 2001;39(6):1523-1532.

54. Ferreira RM, de Andrade LR, Dutra MB, de Souza MF, Flosi Paschoalin VM, Silva JT. Purification and characterization of the chaperone-like Hsp26 from Saccharomyces cerevisiae. Protein expression and purification 2006;47(2):384-392.

55. Abbas-Terki T, Donze O, Briand PA, Picard D. Hsp104 interacts with Hsp90 cochaperones in respiring yeast. Molecular and cellular biology 2001;21(22):7569-7575.

56. Lum R, Tkach JM, Vierling E, Glover JR. Evidence for an unfolding/threading mechanism for protein disaggregation by Saccharomyces cerevisiae Hsp104. The Journal of biological chemistry 2004;279(28):29139-29146.

57. Bosl B, Grimminger V, Walter S. The molecular chaperone Hsp104--a molecular machine for protein disaggregation. Journal of structural biology 2006;156(1):139-148.

58. Bosl B, Grimminger V, Walter S. Substrate binding to the molecular chaperone Hsp104 and its regulation by nucleotides. The Journal of biological chemistry 2005;280(46):38170-38176.

59. Chernoff YO. Stress and prions: lessons from the yeast model. FEBS letters 2007;581(19):3695-3701.

60. Shorter J, Lindquist S. Prions as adaptive conduits of memory and inheritance. Nature reviews 2005;6(6):435-450.

61. Vacher C, Garcia-Oroz L, Rubinsztein DC. Overexpression of yeast hsp104 reduces polyglutamine aggregation and prolongs survival of a transgenic mouse model of Huntington's disease. Human molecular genetics 2005;14(22):3425-3433.

62. Quan X, Tsoulos P, Kuritzky A, Zhang R, Stochaj U. The carrier Msn5p/Kap142p promotes nuclear export of the hsp70 Ssa4p and relocates in response to stress. Molecular microbiology 2006;62(2):592-609.

63. Tkach JM, Glover JR. Nucleocytoplasmic trafficking of the molecular chaperone Hsp104 in unstressed and heat-shocked cells. Traffic (Copenhagen, Denmark) 2008;9(1):39-56.

64. Ryan KJ, Zhou Y, Wente SR. The karyopherin Kap95 regulates nuclear pore complex assembly into intact nuclear envelopes in vivo. Molecular biology of the cell 2007;18(3):886-898.

65. Flom G, Weekes J, Johnson JL. Novel interaction of the Hsp90 chaperone machine with Ssl2, an essential DNA helicase in Saccharomyces cerevisiae. Current genetics 2005;47(6):368-380.

66. Liu XD, Morano KA, Thiele DJ. The yeast Hsp110 family member, Sse1, is an Hsp90 cochaperone. The Journal of biological chemistry 1999;274(38):26654-26660.

67. Longtine MS, McKenzie A, 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast (Chichester, England) 1998;14(10):953-961.

68. Mumberg D, Muller R, Funk M. Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. Nucleic acids research 1994;22(25):5767-5768.

69. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nature biotechnology 2004;22(12):1567-1572.

70. Bohen SP, Yamamoto KR. Isolation of Hsp90 mutants by screening for decreased steroid receptor function. Proceedings of the National Academy of Sciences of the United States of America 1993;90(23):11424-11428.

71. Keppler-Ross S, Noffz C, Dean N. A new purple fluorescent color marker for genetic studies in Saccharomyces cerevisiae and Candida albicans. Genetics 2008;179(1):705-710.

72. Xu Y, Lindquist S. Heat-shock protein hsp90 governs the activity of pp60v-src kinase. Proceedings of the National Academy of Sciences of the United States of America 1993;90(15):7074-7078.

73. Swisher KD, Parker R. Localization to, and effects of Pbp1, Pbp4, Lsm12, Dhh1, and Pab1 on stress granules in Saccharomyces cerevisiae. PloS one;5(4):e10006.

74. Shaner L, Trott A, Goeckeler JL, Brodsky JL, Morano KA. The function of the yeast molecular chaperone Sse1 is mechanistically distinct from the closely related hsp70 family. The Journal of biological chemistry 2004;279(21):21992-22001.

75. Kimura Y, Matsumoto S, Yahara I. Temperature-sensitive mutants of hsp82 of the budding yeast Saccharomyces cerevisiae. Mol Gen Genet 1994;242(5):517-527.

76. Ooi CE, Rabinovich E, Dancis A, Bonifacino JS, Klausner RD. Copper-dependent degradation of the Saccharomyces cerevisiae plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. The EMBO journal 1996;15(14):3515-3523.

77. Wright R. Transmission electron microscopy of yeast. Microscopy research and technique 2000;51(6):496-510.

78. Tapia H, Morano KA. Hsp90 nuclear accumulation in quiescence is linked to chaperone function and spore development in yeast. Molecular biology of the cell;21(1):63-72.

79. Nathan DF, Vos MH, Lindquist S. In vivo functions of the Saccharomyces cerevisiae Hsp90 chaperone. Proc Natl Acad Sci U S A 1997;94(24):12949-12956.

80. Herman PK. Stationary phase in yeast. Current opinion in microbiology 2002;5(6):602-607.

81. Werner-Washburne M, Braun E, Johnston GC, Singer RA. Stationary phase in the yeast Saccharomyces cerevisiae. Microbiol Rev 1993;57(2):383-401.

82. Werner-Washburne M, Braun EL, Crawford ME, Peck VM. Stationary phase in Saccharomyces cerevisiae. Mol Microbiol 1996;19(6):1159-1166.

83. Caplan AJ, Cyr DM, Douglas MG. YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. Cell 1992;71(7):1143-1155.

84. Richter K, Walter S, Buchner J. The Co-chaperone Sba1 connects the ATPase reaction of Hsp90 to the progression of the chaperone cycle. Journal of molecular biology 2004;342(5):1403-1413.

85. Mosammaparast N, Pemberton LF. Karyopherins: from nuclear-transport mediators to nuclear-function regulators. Trends in cell biology 2004;14(10):547-556.

86. Rexach M, Blobel G. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell 1995;83(5):683-692.

87. Enenkel C, Blobel G, Rexach M. Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. The Journal of biological chemistry 1995;270(28):16499-16502.

88. Zaman S, Lippman SI, Zhao X, Broach JR. How Saccharomyces responds to nutrients. Annual review of genetics 2008;42:27-81.

89. Morano KA, Thiele DJ. The Sch9 protein kinase regulates Hsp90 chaperone complex signal transduction activity in vivo. The EMBO journal 1999;18(21):5953-5962.

90. Toda T, Cameron S, Sass P, Wigler M. SCH9, a gene of Saccharomyces cerevisiae that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. Genes & development 1988;2(5):517-527.

91. Mollapour M, Tsutsumi S, Donnelly AC, Beebe K, Tokita MJ, Lee MJ, Lee S, Morra G, Bourboulia D, Scroggins BT, Colombo G, Blagg BS, Panaretou B, Stetler-Stevenson WG, Trepel JB*, et al.* Swe1Wee1-dependent tyrosine phosphorylation of Hsp90 regulates distinct facets of chaperone function. Molecular cell;37(3):333-343.

92. Mollapour M, Tsutsumi S, Neckers L. Hsp90 phosphorylation, Wee1, and the cell cycle. Cell cycle (Georgetown, Tex;9(12).

93. Kang KI, Devin J, Cadepond F, Jibard N, Guiochon-Mantel A, Baulieu EE, Catelli MG. In vivo functional protein-protein interaction: nuclear targeted hsp90 shifts cytoplasmic steroid receptor mutants into the nucleus. Proceedings of the National Academy of Sciences of the United States of America 1994;91(1):340-344.

94. Harrell JM, Murphy PJ, Morishima Y, Chen H, Mansfield JF, Galigniana MD, Pratt WB. Evidence for glucocorticoid receptor transport on microtubules by dynein. The Journal of biological chemistry 2004;279(52):54647-54654.

95. Neiman AM. Ascospore formation in the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev 2005;69(4):565-584.

96. Primig M, Williams RM, Winzeler EA, Tevzadze GG, Conway AR, Hwang SY, Davis RW, Esposito RE. The core meiotic transcriptome in budding yeasts. Nature genetics 2000;26(4):415-423.

97. Xu Y, Singer MA, Lindquist S. Maturation of the tyrosine kinase c-src as a kinase and as a substrate depends on the molecular chaperone Hsp90. Proceedings of the National Academy of Sciences of the United States of America 1999;96(1):109-114.

98. Bohen SP. Hsp90 mutants disrupt glucocorticoid receptor ligand binding and destabilize aporeceptor complexes. The Journal of biological chemistry 1995;270(49):29433-29438.

99. Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proceedings of the National Academy of Sciences of the United States of America 1994;91(18):8324-8328.

100. Bohen SP. Genetic and biochemical analysis of p23 and ansamycin antibiotics in the function of Hsp90-dependent signaling proteins. Molecular and cellular biology 1998;18(6):3330-3339.

101. Krisak L, Strich R, Winters RS, Hall JP, Mallory MJ, Kreitzer D, Tuan RS, Winter E. SMK1, a developmentally regulated MAP kinase, is required for spore wall assembly in Saccharomyces cerevisiae. Genes & development 1994;8(18):2151-2161.

102. Yamaguchi H, Morita T, Amagai A, Maeda Y. Changes in spatial and temporal localization of Dictyostelium homologues of TRAP1 and GRP94 revealed by immunoelectron microscopy. Exp Cell Res 2005;303(2):415-424.

103. Werner-Washburne M, Stone DE, Craig EA. Complex interactions among members of an essential subfamily of hsp70 genes in Saccharomyces cerevisiae. Molecular and cellular biology 1987;7(7):2568-2577.

104. Becker J, Craig EA. Heat-shock proteins as molecular chaperones. European journal of biochemistry / FEBS 1994;219(1-2):11-23.

105. Vogel JL, Parsell DA, Lindquist S. Heat-shock proteins Hsp104 and Hsp70 reactivate mRNA splicing after heat inactivation. Curr Biol 1995;5(3):306-317.

106. Sheth U, Parker R. Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science (New York, NY 2003;300(5620):805-808.

107. Parker R, Sheth U. P bodies and the control of mRNA translation and degradation. Molecular cell 2007;25(5):635-646.

108. Buchan JR, Parker R. Eukaryotic stress granules: the ins and outs of translation. Molecular cell 2009;36(6):932-941.

109. Kedersha N, Stoecklin G, Ayodele M, Yacono P, Lykke-Andersen J, Fritzler MJ, Scheuner D, Kaufman RJ, Golan DE, Anderson P. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. The Journal of cell biology 2005;169(6):871-884.

110. Buchan JR, Muhlrad D, Parker R. P bodies promote stress granule assembly in Saccharomyces cerevisiae. The Journal of cell biology 2008;183(3):441-455.

111. Nissan T, Parker R. Analyzing P-bodies in Saccharomyces cerevisiae. Methods in enzymology 2008;448:507-520.

112. Decker CJ, Teixeira D, Parker R. Edc3p and a glutamine/asparagine-rich domain of Lsm4p function in processing body assembly in Saccharomyces cerevisiae. The Journal of cell biology 2007;179(3):437-449.

113. Reijns MA, Alexander RD, Spiller MP, Beggs JD. A role for Q/N-rich aggregation-prone regions in P-body localization. Journal of cell science 2008;121(Pt 15):2463-2472.

114. Bracken AP, Bond U. Reassembly and protection of small nuclear ribonucleoprotein particles by heat shock proteins in yeast cells. RNA (New York, NY 1999;5(12):1586-1596.

115. Yost HJ, Lindquist S. Heat shock proteins affect RNA processing during the heat shock response of Saccharomyces cerevisiae. Molecular and cellular biology 1991;11(2):1062-1068.

116. Rossi JJ. RNAi and the P-body connection. Nature cell biology 2005;7(7):643-644.

117. Iwasaki S, Kobayashi M, Yoda M, Sakaguchi Y, Katsuma S, Suzuki T, Tomari Y. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. Molecular cell;39(2):292-299.

118. Toogun OA, Dezwaan DC, Freeman BC. The hsp90 molecular chaperone modulates multiple telomerase activities. Molecular and cellular biology 2008;28(1):457-467.

119. Toogun OA, Zeiger W, Freeman BC. The p23 molecular chaperone promotes functional telomerase complexes through DNA dissociation. Proceedings of the National Academy of Sciences of the United States of America 2007;104(14):5765-5770.

120. Floer M, Bryant GO, Ptashne M. HSP90/70 chaperones are required for rapid nucleosome removal upon induction of the GAL genes of yeast. Proceedings of the National Academy of Sciences of the United States of America 2008;105(8):2975-2980.

121. Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood) 2003;228(2):111-133.

122. Shulga N, James P, Craig EA, Goldfarb DS. A nuclear export signal prevents Saccharomyces cerevisiae Hsp70 Ssb1p from stimulating nuclear localization signal-directed nuclear transport. The Journal of biological chemistry 1999;274(23):16501-16507.

123. Gray JV, Petsko GA, Johnston GC, Ringe D, Singer RA, Werner-Washburne M. "Sleeping beauty": quiescence in Saccharomyces cerevisiae. Microbiol Mol Biol Rev 2004;68(2):187-206.

124. Shapiro RS, Uppuluri P, Zaas AK, Collins C, Senn H, Perfect JR, Heitman J, Cowen LE. Hsp90 orchestrates temperature-dependent Candida albicans morphogenesis via Ras1-PKA signaling. Curr Biol 2009;19(8):621-629.

125. Hung GC, Masison DC. N-terminal domain of yeast Hsp104 chaperone is dispensable for thermotolerance and prion propagation but necessary for curing prions by Hsp104 overexpression. Genetics 2006;173(2):611-620.

126. Duttagupta R, Vasudevan S, Wilusz CJ, Peltz SW. A yeast homologue of Hsp70, Ssa1p, regulates turnover of the MFA2 transcript through its AU-rich 3' untranslated region. Molecular and cellular biology 2003;23(8):2623-2632.

127. Collier NC, Schlesinger MJ. The dynamic state of heat shock proteins in chicken embryo fibroblasts. The Journal of cell biology 1986;103(4):1495-1507.

VITA

Hugo Tapia was born in Ciudad Juarez, Chihuhua Mexico on July 26, 1981 the son of Socorro del Real and Bernardino Tapia. After graduating from Riverside High School in El Paso, Texas in 1999, he enrolled at the University of Texas at El Paso. He earned his Bachelor of Science in Microbiology with a minor in Chemistry from the University of Texas at El Paso in May of 2004. In August of 2004 he entered the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. In the summer of 2005 he joined the laboratory of Dr. Kevin A. Morano.